THERMOSTABILIZATION OF INFLUENZA VACCINE

IN MICRONEEDLE PATCHES

A Thesis
Presented to
The Academic Faculty

by

Matthew Joseph Mistilis

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in the
School of Chemical and Biomolecular Engineering

Georgia Institute of Technology
May 2016

THERMOSTABILIZATION OF INFLUENZA VACCINE

IN MICRONEEDLE PATCHES

Approved by:

Dr. Mark R. Prausnitz, Advisor
School of Chemical and Biomolecular Engineering
Georgia Institute of Technology

Dr. Andreas Bommarius, Advisor
School of Chemical and Biomolecular Engineering
Georgia Institute of Technology

Dr. Julie Champion
School of Chemical and Biomolecular Engineering
Georgia Institute of Technology

Dr. Richard Compans
Department of Microbiology and Immunology
Emory University

Dr. Raquel Lieberman
School of Chemistry and Biochemistry
Georgia Institute of Technology

Date Approved: December 11, 2015

To my family

ACKNOWLEDGEMENTS

A single person earns a doctoral degree, but it is anything but a solo effort. There are of course many people who have contributed along the way to this accomplishment. From the very beginning, Dr. Mark Prausnitz has helped me to find my way academically, scientifically, and professionally. Working with him has been a great honor. He has pushed me to maximize my potential and keep the big picture in mind in an environment seemingly designed to bog people down. Completing a Ph.D. program is multifaceted. The most obvious task is to become a subject matter expert on whatever your project focuses on, but often overlooked aspects are to develop the skills to think ahead, to tackle open-ended, daunting challenges, and to clearly communicate the importance of your work to various audiences. In every way, Dr. Prausnitz has been there to mentor me in every aspect of my path to a Ph.D.

My co-advisor, Dr. Andy Bommarius, has been invaluable asset for me, especially once he became more involved as a co-advisor instead of a committee member. It's usually very helpful to have multiple points of view, and this case was no different. As one of only a few people related to this project who does not work on microneedle technology, his input on the protein-specific studies and interpretation have helped immensely. Not only has Dr. Bommarius himself been valuable, so has his lab, who took me in and taught me new techniques and tricks when working with proteins. Among others in his lab, Dr. Bettina Bommarius, Dr. Mick Robbins, Sam Au, and Aditi Sharma were particularly helpful. I would also like to thank the rest of my thesis committee, Dr. Julie Champion, Dr. Richard Compans, and Dr. Raquel Lieberman, for their time and effort to advise me along the way with always helpful ideas, comments, and questions.

Those that work in Dr. Prausnitz's lab recognize that our productivity would be a mere fraction of what it is now without our Program & Operations Manager, Donna Bondy. She handles much of the day-to-day business surrounding our work here, and we would all be lost without her. I can't say how appreciative I am of her help, from ordering essential supplies and reimbursing travel expenses to brightening up the office area when she stops to chat with us. Thank you, Donna.

To put into perspective how fantastic the people in the Drug Delivery Lab are, when most people go home for vacation, they don't miss their coworkers and look forward to returning to work just to spend more time with them. I've felt this several times, and I am very thankful for the environment I get to work in 5-7 days a week. Spending time together outside of lab was relatively common, including game nights, potluck dinners, Diwali celebrations, roller skating, and a Braves game. Our lab's 2015 retreat to Destin, Florida was a good mix of work and pleasure, and we all fit well together.

More specifically, I'd like to thank Dr. Vladimir Zarnitsyn for setting up the coating station and helping me out when I first started. I would like to thank Brian Bondy for teaching me how to make dried vaccine chip samples and test their activity with a hemagglutination assay. I would like to thank Dr. Priya Kalluri for developing activity assays with me and advice on vaccine formulations. I would like to thank Dr. Devin McAllister and Winston Pewin for their fantastic work developing microneedle patch production methods. I would like to thank Dr. Jeong Woo Lee for many consultations about issues such as assay troubleshooting and various microneedle patch production methods. I would like to thank Jessica Joyce for helpful discussions of vaccine and immunological matters as well as assistance with my in vivo study. I would like to thank Dr. Ioanna

Skountzou and Stein Esser, both from Emory University for further assistance with planning my in vivo study as well as serum analysis. I would like to thank Dr. James Norman and Bryce Chiang for being my stats stars. I can show them a collection of data and they would have advice on what statistical test to run and what all the parameters mean in real world English. I would like to thank Dr. Xin Dong Guo for creation of the master molds I used for microneedle patch production. I would like to thank my two undergraduate research assistants, Polo Gaputan and Miraj Desai. They did a lot of the leg work in preparing samples and analyzing the resulting data. They both helped things progress at a much faster pace. While I've pointed out these individuals, I would be remiss to leave out others from the Drug Delivery Lab that I am very thankful for the opportunity to work with. These people include, but are not limited to, Andrew Tadros, Dr. Andrey Romanyuk, Dr. Aritra Sengupta, Chandana Kolluru, Dr. Chris Edens, Dr. Han Jung Park, Dr. Hong Wei Yang, Jaya Arya, Pradnya Samant, Dr. Samir Patel, Sebastien Henry, Dr. Seong-O Choi, Dr. Seonhee Park, Stefany Holguin, Dr. Wilmarie Medina-Ramos, Dr. Yasmine Gomaa, Dr. Yeu Chun, Dr. Yoo Chun. Other people who have been a great help are Dr. Hong Yi from the Robert P. Apkarian Integrated Electron Microscopy Core at Emory University and Dr. Shannon Hill from Dr. Lieberman's lab at Georgia Tech.

Last, but certainly not least, I must thank my friends and family who have been my support system throughout it all. My parents, Joey and Cathie, along with my sister Lauren, have never lost faith in my pursuit of further education and have supported me as long as I can remember, and probably before that point. Their care and love have been a constant in my life, which I'm extremely grateful for. My entire family has always had my back. I've also always had great friends on my side as well. The friends I've had have of course made

this journey much more enjoyable and memorable. One group in particular, MATLAB ME, as we like to call ourselves, have helped keep me sane throughout graduate school. An agglomeration of Matt, Abi Shitta, Timi Fadiran, Lindsay Arnold, Andy Peters, Brennen Mueller, and Maria Elena Casas, we've have made it a long way from a first-year study group to a bunch of kids with Ph.D.'s. There's clearly a lot of people that have gone into this one PhD, but that's what makes it fun.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

Vaccines have long been delivered with a hypodermic needle and syringe. Several drawbacks of this method include patient pain, biohazardous sharps waste, and the need for administration by a healthcare worker. Aside from the delivery method, vaccines are also relatively unstable outside of a controlled cold-chain of refrigeration and freezer equipment. Most current vaccines are stored either refrigerated at 2-8°C or frozen at -20°C throughout storage and transportation, adding substantial cost to immunization programs from both the maintenance of a controlled cold-chain as well as vaccine wastage when that cold-chain fails. A novel drug delivery method currently being developed, called a microneedle patch, could possibly mitigate all of these disadvantages.

Microneedle patches are composed of submillimeter projections, called microneedles (MNs), arranged in an array on a solid backing. These MNs are composed of water soluble excipients and polymers with the drug encapsulated inside the MN. When the MNs are inserted into skin, they dissolve in the interstitial fluid and release their payload. The backing is then removed from the skin, and the procedure is complete. There is no sharps waste generated with this painless delivery, and it may be possible for patients to self-administer their patches. Also, MN patches exist in a dried state, possibly lending itself to the stability of any vaccine inside of it.

The first aim of this work was to screen possible MN formulations to maintain influenza vaccine antibody-binding activity during air drying on flat surfaces representative of the production process and during subsequent storage without refrigeration. Initial work began from established coated MN formulations developed previously with 15% w/v trehalose and 1% w/v carboxymethylcellulose. Vaccine was buffer exchanged into various buffer solutions and dried at three different temperatures. In general, drying quickly at 40°C caused slightly more vaccine activity loss compared to drying at 4°C or 25°C. To simplify later testing, 25°C was chosen as the drying temperature because it was not detrimental to stability. Phosphate-buffered saline, while suitable for many aqueous proteins, led to a significant loss of activity after drying for a short time. Two buffers, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and

ammonium acetate, both performed well, and ammonium acetate was chosen as the buffer for all subsequent formulation studies. Surfactants, while helpful for creating a coated MN, were not found to have an effect on vaccine activity during initial drying, and were thus omitted from further formulations. A large number of compounds were screened for stability, with the majority of good performing stabilizers being carbohydrates, with the noted exceptions of arginine and histidine. Previous evidence from work with dry measles formulation suggested a combination of stabilizers could be beneficial, so combinations of top stabilizers were tested for up to one month at 40°C. Three formulations were found to have no statistically significant loss of activity throughout the month of storage as well as no activity loss at 40°C for up to eighteen months. These formulations were 50/50 combinations of trehalose/sucrose, sucrose/arginine, and arginine/heptagluconate. They were then implemented into the production of influenza vaccine microneedle patches.

Both monovalent and trivalent influenza vaccine microneedles were produced with the three top stabilizer combinations. These patches were shown to be stable for up to eighteen months at 25°C, which is more prolonged than would need be for annual seasonal influenza vaccine patches. A set of patches with the stabilizer combination of arginine/heptagluconate that had shown full stability after 12 months was used to vaccinate BALB/c mice. Compared to comparable doses of vaccine delivered intradermally with a needle and syringe, both aged and fresh patches elicited a higher antibody response. To remove the effect of delivery method, some aged patches were reconstituted in buffer and delivered intradermally as well. These showed comparable antibody titers to the same dose of fresh vaccine delivered intradermally. As added measures of vaccine stability in microneedle patches, patches formulated with arginine and heptagluconate were subjected to freeze-thaw cycles, 60°C storage for four months, and electron beam irradiation. None of these conditions caused a statistically significant loss of vaccine activity.

For a deeper look into influenza vaccine stability during drying, the mechanism of activity loss was investigated with a number of protein analytical techniques. Dynamic light scattering (DLS) and transmission electron microscopy both showed multimeric rosettes typical of subunit vaccines as well as larger spherical protein structures. While the size and frequency of these larger structures were not noticeably different between liquid and dried vaccine when analyzed by transmission electron microscopy (TEM), DLS did

show a more chaotic size distribution for dried vaccine. This phenomenon was prevented by the inclusion of trehalose in the vaccine solution before drying. Protein structure changes were also probed with spectroscopic techniques. Circular dichroism spectroscopy and intrinsic protein fluorescence spectroscopy essentially showed no structural changes, and the few changes that were found, while statistically significant, were not deemed to be the cause of vaccine activity loss. Therefore the predominant mechanism of influenza vaccine activity loss was aggregation.

Overall, this work sought to develop a dissolving MN patch that can preserve subunit influenza vaccine *in vitro* activity and *in vivo* immunogenicity. MN patches developed here have been shown to be both robust against several potentially harsh conditions and maintained vaccine activity throughout long-term storage outside of the cold-chain. These same aged patches generated seropositive antibody titers in mice. Finally, it was determined that the primary mechanism of vaccine activity loss during drying is aggregation. We foresee MN patches help to increase vaccination coverage through higher patient compliance and a cheaper, more immunogenic, and more stable delivery method.

CHAPTER I

INTRODUCTION

The introduction of vaccines has seen a dramatic drop in many diseases that once caused widespread human death and suffering. They teach the body to fight infections before the disease causing agent can cause too much harm. The most widespread method to deliver these vaccines is a hypodermic needle and a syringe. Vaccine delivery to the skin via microneedles confers several advantages over the traditional hypodermic needle and syringe. This work focuses on developing microneedles as a thermostable delivery method for the influenza vaccine that can be completely removed from the cold-chain, thus minimizing cost and wastage during storage and transportation. This removal from the cold chain will allow for cheaper and more wide-spread vaccination campaigns. The development and commercialization of microneedle patches could improve overall vaccination coverage in both developed and developing countries, eliminated much of the suffering and economic costs related to vaccine-preventable illnesses.

CHAPTER II

BACKGROUND

2.1 Influenza virus and vaccine

The disease known as the flu is caused by the influenza virus, an RNA virus of the *Orthomyxoviridae* family.[1] These typically spherical or filamentous virus particles consist of 8 strains of RNA, which encode a total of 11 proteins.[2] Two of these proteins are hemagglutinin and neuraminidase, both of which are surface membrane proteins that are believed to be the basis of human immunological responses.[3] Hemagglutinin (HA) is a glycoprotein responsible for attachment to the host cell as well as membrane fusion with the host cell to facilitate introduction of viral cargo.[4] Neuraminidase (NA) is also a glycoprotein which facilitates the release of newly formed virus particles by cleaving the sialic acid sites of the cell membrane.[5]

The 3 different genera of influenza are known as influenza virus A, influenza virus B, and influenza virus C.[5] Although humans can be infected by influenza C, usually during early childhood, it is much rarer than infection by influenza A or B.[6] Current vaccines consist of two strains of influenza A and one or two strains of influenza B.[7] The primary antigen that the influenza vaccine is based on is HA.[8] Typical seasonal influenza vaccines contain roughly 15 μg of HA from each of the three strains predicted by the World Health Organization to best match the circulating strains for a given year.[9] The strains chosen include two influenza A strains and one influenza B strain, although a quadrivalent influenza vaccine which contains a second influenza B strain has been approved in the United States.[10]

HA is a 225 kDa homotrimer glycoprotein formed from three 75 kDa monomers, each of which is composed of two subunits, HA1 and HA2 (Figure 2.1).[5] The specific amino acid sequence of HA is dependent on the strain of influenza, but the overall structure is relatively unchanged across strains.[11] HA is synthesized within the host cell as a non-fusogenic precursor, HA0, before being glycosylated and cleaved to create the fusogenic

form, where HA1 and HA2 are connected by a disulfide bond.[12] The overall structure of HA is an elongated coiled-coil stalk with a large globular head.[13] HA's roles in infection are performed by the two subunits on each monomer. The large globular head, derived from HA1, is responsible for binding to sialic acid on the target membrane.[14] The central triple-stranded coiled-coil stalk, derived from HA2, is responsible for fusion between the host membrane and viral membrane, thus creating a pore through which the viral payload can infect the target cell.[15] This fusion region, though, is not typically accessible before HA undergoes a low-pH induced conformational change after sialic acid binding and endocytosis.[16] This conformational change involves the globular head moving to the side of the protein while the central stock undergoes a refolding that exposes the membrane fusion region.[17] This conformational change has also been known to be caused by heat or urea treatment.[18]

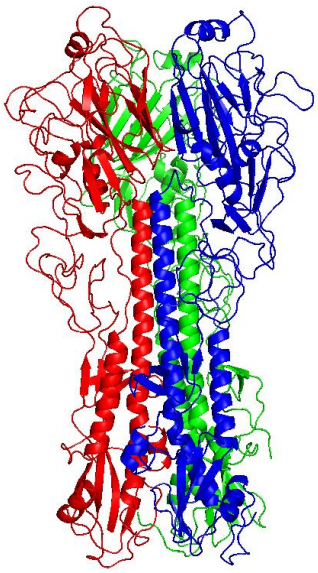

Figure 2.1. A cartoon representation of the ectodomain of influenza hemagglutinin. The strain depicted is from the B/Brisbane/60/2008 with each color representing a different monomer. The top of the illustration is the membrane distal region. Used with permission.[19]

Current influenza vaccines generally are administered as one of 5 types of antigen particles: whole inactivated vaccine (WIV), live attenuated influenza vaccine (LAIV), virosomal vaccine, split virus vaccine, and subunit vaccine.[20] The first three types include an intact virus membrane. A whole inactivated vaccine is a virus particle that has been treated with formaldehyde[21] or β-propiolactone[22] such that the RNA is not able to be replicated inside the host cell.[22] The live attenuated vaccine is whole virus particles that have been cold-adapted by repeated passage through chicken eggs at decreasing temperatures.[23] The virus particles can then only survive in the nasal cavity and not in any internal organs.[24] Thus it elicits a mucosal immune response without a systemic infection.[23] The virosomal vaccine, such as Influvac® and Inflexal®, from Abbott Laboratories and Crucell Vaccines Inc., respectively, is also an intact membrane vesicle similar to WIV and LAIV. Instead of a modified whole virus, though, it is reconstituted from subunit particles, such as partial influenza envelopes, so that the interior of the membrane is void of genetic material.[25] Both split virus vaccines and subunit vaccines are manufactured by breaking whole virus particles into many smaller pieces such as RNA, intracellular proteins, and pieces of membrane with attached membrane proteins. However, a subunit vaccine has been purified to only contain pieces of membrane with attached membrane proteins.[26] This is due to the belief that the HA and NA proteins are what elicit human immune responses.[20] Owing to the aggregation of their hydrophobic transmembrane regions, HA from split virus and subunit vaccines are mostly found as multiunit rosettes.[27]

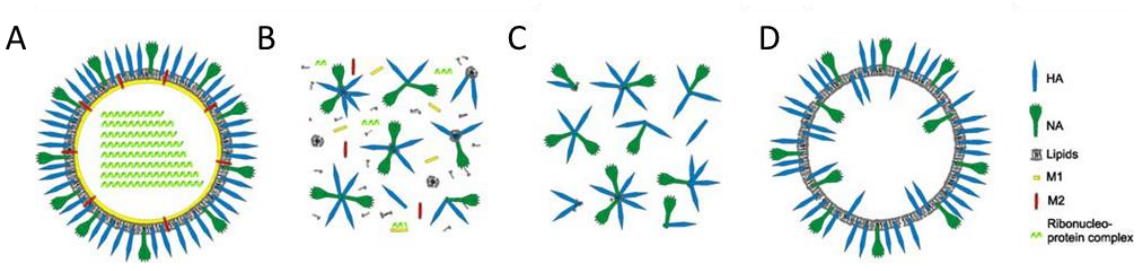

Figure 2.2. Representative cartoons of various influenza vaccine types: (A) whole-inactivated or live attenuated, (B) split virus, (C) subunit, (D) virosomal. Used with permission.[20]

2.2 Current methods of vaccine delivery

There are several routes of influenza vaccination currently being utilized. The most common method is an intramuscular (IM) injection using a syringe and a hypodermic needle. An aqueous suspension of vaccine, possibly along with any preservatives and adjuvants, are injected directly into muscle tissue. An adjuvant is a compound which helps boost the body's immunological response to an antigen.[28] Possible modes of action of an adjuvant are immunomodulation, antigen presentation, cytotoxic T-lymphocyte induction, antigen presenting cell targeting, or depot formation.[29] Another similar method is subcutaneous (SC) injections. SC injections deposit the vaccine suspension directly in the fatty tissue directly beneath the dermal layer.[30] Intradermal (ID) injections delivery vaccine within the dermis layer of the skin.[31] This can be advantageous because of the higher density of antigen presenting cells, such as dermal dendritic cells and Langerhans cells.[32]

Although a hypodermic needle and syringe is the most common form of vaccine delivery, there are drawbacks to all three major hypodermic injections. It is required that healthcare personnel administer the vaccine, and even with the training healthcare personnel receive, needle-stick injuries sometimes occur.[33] These can lead to the exposure to unknown pathogens and the transmission of blood-borne infectious diseases.[34] Since ID injections need to be more precise than IM or SC injections, even more training and skills are required for the person administering the dose.[35]

Any route of vaccination using a needle and syringe has similar drawbacks. As stated, needle-stick injuries can introduce other pathogens to personnel, which can lead to transmission of infectious diseases.[36] Patients can be hesitant to receive any treatment via an injection due to the pain of injection and needle-phobia. This decreases patient compliance.[37] Disposing of used needles presents a hazard because they must be disposed of safely as sharps biohazard waste.

There are other, more novel routes of influenza vaccine. FluMist is an FDA-approved intranasal delivery suspension/device. Currently being manufactured and marketed by MedImmune[38], it painlessly delivers an aerosolized LAIV mist directly to the nasal cavity to elicit an immune response.

Delivery methods are also being developed for intradermal[35, 39, 40] and transdermal[41, 42] routes. As of the 2011-2012 influenza season, an ID influenza vaccine has been approved and available in the United States.[43] Fluzone Intradermal, by Sanofi Pasteur, is available to adults 18 to 64 years of age and is delivered through a needle much smaller than a typical hypodermic needle.[43] The needle used is 1.5 mm in length, which is 90% shorter than other needles used for IM influenza vaccine delivery, and is used to deliver a 0.1 mL dose of trivalent vaccine.[44] It was found in a Phase III trial that a reduced dose delivered intradermally using a microinjection system provided comparable hemagglutination inhibition levels as the standard trivalent vaccine delivered intramuscularly.[45] Also approved for use in the United States, PharmaJet® Inc. utilizes a needleless jet injector to delivery bioCSL Inc.'s AFLURIA inactivated influenza vaccine into the skin.[46] The jet injector system allows for vaccine delivery without the generation of biohazardous sharps waste, but has been found to have a higher incidence rate of infection.[47]

2.3 Microneedle basics

A new method is being developed for delivery of vaccines into skin. This method uses solid projections called microneedles (MN). Each MN is less than 1 millimeter in length, and they are most often placed together with other MNs to form what is called a "patch". The MNs are designed to be long enough so that they pass through the tough outer layer of the epidermis, called the stratum corneum, but short enough so that they do not contact the skin's blood vessels or nerves. This can lead to an improved immune response compared to an IM injection[40, 48] with the nearly painless insertion of a MN patch[49]. The improved immune response is thought to be a result of a large population of immune cells in the skin, such as Langerhans cells and dermal dendritic cells.[50, 51] It has been shown that MN vaccinations, when compared to IM vaccinations, induce cellular and humoral immune responses that by some measures are significantly greater.[52]

There are several different varieties of MN patches (Figure 2.3). The simplest one is essentially a shorter version of a standard needle. These hollow MNs have a small-diameter bore with a sharp tip and are designed to be long enough to pass just through the stratum corneum and into the epidermis and dermis.[53] A small volume of drug solution

can then be injected into the skin for either bolus or extended delivery.[54] The other type of MN that does not have any active pharmaceutical ingredient (API) incorporated directly with the design is the solid MN patch. These MNs, containing no API, are composed of solid metal[55, 56], polymer[57], or silicon[58] projections designed to insert through skin to disrupt the stratum corneum. After the MNs are fully inserted, they are removed to leave pores in the skin. Gels, creams, or passive transdermal patches are then placed on the skin over the pores so that the (API), which wouldn't normally be able to diffuse well into the skin, can be delivered below the stratum corneum. This method is typically referred to as "poke and patch" delivery.

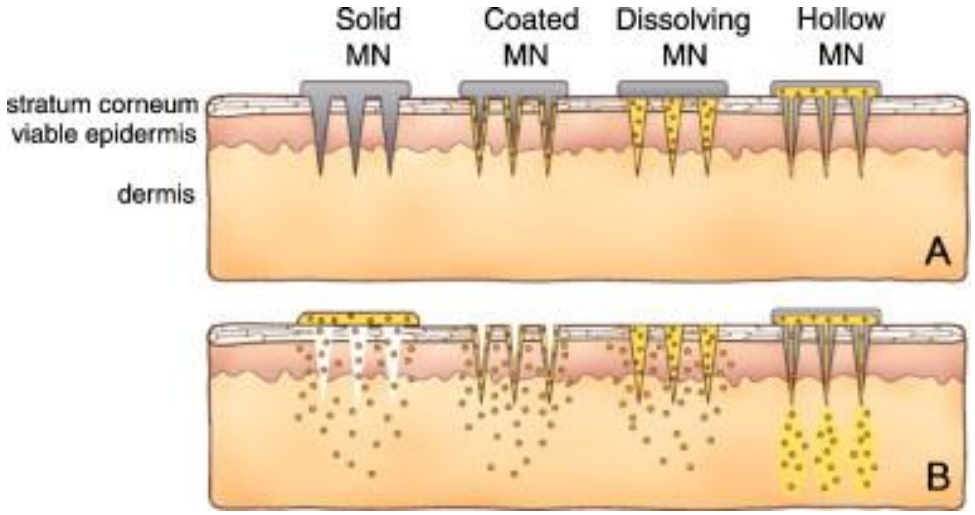

Figure 2.3. Four different types of microneedle patches. (A) Microneedle patches upon initial insertion into skin. (B) Drug being delivered across the stratum corneum into the dermis and epidermis. Used with permission.[59]

Another variety is the coated MN patch. Here a metal projection, made of metal[60, 61] or silicon[62, 63], is either cut out of a thin, flat sheet with laser ablation or chemical etching. After electropolishing and cleaning, the metal MNs are ready to be coated with the API to be delivered. This is done by repeated dip-coating into a solution of the API and any other formulation compounds that constitute the coating solution.[64] For many applications, a surfactant is necessary to help wet the entire hydrophobic metal surface.[65] A viscosity enhancer is also added to the solution to help form a thicker coating, thus allowing more API to be coated on the surface of the MNs.[66] The process

of dip-coating lends itself to automation by controlling the movement of either the coating solution reservoir or the MN arrays.[60] Once the MN patch has been coated, it can then be inserted into the skin where the needles penetrate into the epidermis. Once there, the coating dissolves and releases the API payload that it contains to be acted upon by the skin's rich immune system environment.[67]

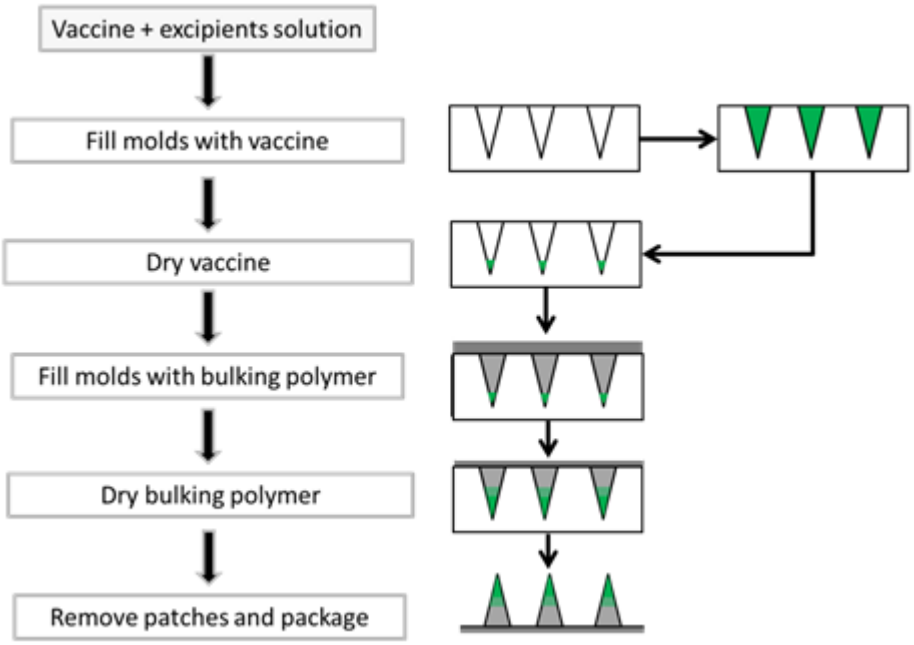

Figure 2.4. Flowchart of the dissolving microneedle patch production process.

The last type of MN covered here is the dissolving MN patch. Each MN is a solid projection comprised primarily of water-soluble polymers and stabilizing excipients.[68] As described by Vassilieva *et.al.*[69], the production method (Figure 2.4) used here is a liquid casting into polydimethylsiloxane (PDMS) molds with the assistance of vacuum. A solution of the API and other excipients, such as stabilizers and polymers, is placed on top of a negative PDMS mold with MN shaped cavities. The mold is the exposed to vacuum to help the solution completely fill the mold, removing a small pocket of air that can occur at the MN tip. Once the mold is filled, the API solution is allowed to dry down into the tips of the MN cavities. The final casting step is to fill the mold with a backing solution, which

will create the rest of each MN as well as a backing to facilitate the demolding of all the MNs together. In this work, this solution contains polyvinyl alcohol and sucrose, both of which are readily soluble in the skin's interstitial fluid. With all of the components in place, the patch is then dried still in the mold with desiccant at room temperature for several days. The MN patch is then demolded and is ready to be used. The process of using a dissolving MN patch is very similar to that of a coated MN patch, except after MN insertion, the entire MN structure dissolves instead of simply a coating. This dissolution releases the API payload as well as ensures there is no sharps waste once the immunization is complete and the MN patch backing is removed from the skin.[70]

2.4 Dry protein stabilization

Viruses and vaccine-related proteins, like most biological structures, are accustomed to being inside a living system and, therefore, prefer to be in an aqueous environment.[71] During the manufacturing of MN patches, though, the environment that the vaccine is in becomes decreasingly aqueous to the point where can be considered dry and no longer aqueous. This change in surroundings can stress the vaccine, possibly to the extent that it is no longer active as the desired antigen.[72] Vaccine antibody-binding activity loss could occur during two different time frames with respect to MN patches. The first is the initial drying while the vaccine is being incorporated into the microneedle patch. The second is during the storage of these patches. Under certain conditions, such as high temperature or humidity, it is possible for the matrix compounds to change their physical state from one of a transient amorphous state to a more thermodynamically stable crystalline state. Thus, a production method and formulation must be developed to help maintain vaccine antigenicity and overall stability during the production and subsequent dry storage of MN patches.

As the protein of interest in this work is an antigen for immunization, stabilization here refers to the ability of the protein to bind to antibodies in *in vitro* assays or to cause the production of antibodies *in vivo*. This ability to activate the immune system and induce the production of neutralizing antibodies determines whether the protein is still immunogenic. This definition of protein stability leaves open the possibility that small

changes, such as oxidation or deamidation, can occur to the protein without affecting its immunogenicity.

One common criterion monitored for protein stability is protein aggregation, which is the interaction and association of multiple proteins.[73] Although aggregation is usually thought of as the association of hydrophobic regions of a protein, there are several pathways that can lead to protein aggregation.[74] Proteins typically have hydrophilic amino acid residues on their outer surfaces with more hydrophobic resides located in the core of the structure, away from their aqueous surrounding.[75] If circumstances occur that the hydrophobic core of a protein is exposed to its aqueous environment, due to events such as misfolding or structural rearrangement, it will then seek to find a more suitable interaction, whether this is a more hydrophobic interface or an exposed region of another protein.[76] Proteins may also self-associate by either electrostatic forces or through hydrophobic interactions without the involvement of an unfolded intermediate although they may involve subtle conformational changes.[74]

The previously mentioned aggregation pathways do not involve chemical changes to the protein's molecular composition. It is possible, though, for proteins to interact after a chemical change. One route is the direct chemical linkage of proteins. While the most common reaction is the breakage and reformation of disulfide bonds[77], it can also include reactions such as dityrosine formation[78], Maillard-type reactions[79], and formaldehyde-mediated cross-linking[80]. Another route for protein aggregation to occur, is by chemical modification of amino acid residue side chains that changes the protein's hydrophobicity, secondary or tertiary structure, or barriers to unfolding. These reactions include common protein modifications such as deamidation, oxidation, deglycosylation, or hydrolysis.[81] All of these chemical modifications could also occur without subsequent aggregation. These would be of interest with regards to vaccine stability if they were to occur in an antigenic region of the protein, thus causing a decrease of immunogenicity.

Changes to the protein stability in an aqueous state can easily become exacerbated when the protein undergoes freezing or drying. In either case, the amount of liquid water proteins and excipients have available to interact with decreases drastically. This increases the concentration of all components in the remaining solution.[82] Several things can result from this concentration. Since aggregation is the interaction or association of multiple

proteins, an increase in protein concentration will hasten this process. The pH of a buffer can also be affected by the concentration of excipients in the protein formulation.[83] An example is phosphate-buffered saline, where the concentration of buffer components leads to a drop in solution pH due to differential precipitation of the buffer components since they all have different solubility limits.[84] This pH change affects protein stability as much as it would in a typical protein solution, although the extent of its effect is also dependent on the rate of freezing or drying. Rapid processing may limit the time that the protein has for changes to occur.[85]

Many lessons for protein stabilization during drying can be found in the extensive work done for protein lyophilization. During the lyophilization process, protein solutions are frozen, placed in a low pressure chamber, and slowly warmed so that the frozen water sublimates directly to water vapor. This results in a product with a very low moisture content. These proteins are often formulated with certain excipients that have been found to help maintain protein stability in the dried state.

These stabilizers are often carbohydrates. There are two theories, which could act together, of how a carbohydrate stabilizes biomolecules, such as proteins[86], liposomes[87], and viruses[88]. One theory involves the vitrification of sugar to form a sugar glass. This sugar glass is an amorphous phase characterized by extremely high viscosity.[89] Biomolecules in this phase are isolated[90] and restrained[91] so that aggregation and interactions are held to a minimum. The other theory is called the water-displacement theory. Biomolecules form many hydrogen bonds with surrounding water molecules. Sugars, which contain hydroxyl groups, can replace these missing hydrogen bonds as water is removed. This helps maintain the environment necessary for the native structure of the biomolecule.[92] Preservation of the amorphous, glassy state is influenced by both storage temperature and overall matrix composition, which is affected by moisture content.[93] The thermodynamic stability of a glass is related to the difference between its storage temperature and its glass transition temperature, known as T_g.[94] This is a well-studied point at which a material transitions from an amorphous, highly viscous glass phase to a higher mobility rubber phase.[95] If an amorphous solid is kept well below its T_g, then it will tend to remain in that state, but if its temperature approaches or surpasses its T_g, then it will transition into a rubbery state.[96] This point is described by the Gordon-Taylor

equation[97], which takes into account the composition of the material as well as the individual T_g of each component. Pure water has a T_g of -137°C[98], therefore small amounts of water in the solid phase lowers the T_g of the solid as a whole. For this reason, the hygroscopicity and ambient humidity of lyophilized products are closely monitored. Sugars which are commonly used as lyoprotectants, such as sucrose and trehalose, have relatively high T_g of 75-115°C[99] and 77°C[100], respectively, although the value depends on the measurement methodology. Thus these sugars help to maintain an amorphous, sugar glass matrix surrounding a biopharmaceutical drug.

While trehalose and sucrose have been used widely in lyophilized biopharmaceutical products, other compounds have been studied and used as stabilizing excipients. Other disaccharides have also been investigated, such as maltose[101] and lactose[102], as well as monosaccharides and larger polysaccharides. A common stabilizing monosaccharide is glucose[103-105], while there are many polysaccharides such as dextran[106, 107] and inulin[108, 109]. The studies involving carbohydrates typically reference the compounds' abilities to hydrogen bond with a protein or to form a glassy phase to immobilize and stabilize a protein. A factor to be aware of when selecting a carbohydrate to include in a formulation is that reducing sugars can lead to protein degradation during storage by Maillard reactions between their free carbonyls and the free amino groups on the protein.[110, 111]

Not all stabilizing compounds must be saccharides, though. Salts can have a large impact on protein stability as well. Salt selection and concentration affect the presence or absence of a hydration shell around a given protein, thus determining whether the protein is "salted out" or "salted in".[112-114] Zhang and Cremer reviewed the order in which salts and osmolytes have salting in or salting out properties, known as the Hofmeister series.[115] Although the exact mechanism of action of salts on proteins in solutions is not well defined, in general, ions that salt out proteins, such as sulfate and carbonate, prevent protein unfolding, thus stabilizing the protein.[116] It should be noted, though, that all formulation components are concentrated as a solution is frozen or dried, and salts can have different effects on proteins depending on their concentration ranges, thus care must be taken when simply choosing a salt at either end of the Hofmeister series.

Non-carbohydrate polymers have also been used to stabilize proteins in solution and in a dried state. The enzyme urokinase has been stabilized by hydroxyl ethyl starch, PEG4000, and gelatin.[117] Polyethylimine was used to stabilize the enzyme lactate dehydrogenase during storage.[118] A common characteristic of protein-stabilizing polymers is their hydrophilic nature.[119] Similar to saccharides' favorable interaction through hydrogen bonding, hydrophilic polymers are able to satisfy electrostatic interactions[120] with the protein surface as well as forming an entangled matrix environment if present in the a dried formulation since many polymers have relatively high glass transition temperature, T_g.[121]

Formulation development for proteins is still relatively empirical. There are several broadly successful excipients and general trends, but the development of a successful formulation is still prepared on a case-by-case basis. There are even more special considerations when working on stabilizing a vaccine formulation because there are many varieties of vaccine. Some vaccines are composed of only proteins, therefore formulation development can utilize the wide range of knowledge about stabilizing proteins. Other vaccines are intact viral particles. Inactivated vaccines must remain intact to retain full immunogenicity, and live attenuated vaccines must remain intact and alive so that they can replicate inside the patient. Altered osmolarity[122] and interfacial surface interactions[123] have been shown to negatively affect vaccine activity in the liquid and dried states.

There are currently no DNA vaccines approved for human use in the United States, although they are in development. DNA vaccine development includes its own set of stability concerns such as DNA rearrangements [124] and nuclease degradation[125], for example. Polysaccharides are also utilized as vaccine components as bacterial capsular polysaccharide antigens.[126] Lone polysaccharides, while more stable than other biomacromolecules, are not strong antigens and therefore are conjugated to carrier proteins to increase antigenicity.[127] Polysaccharide conjugate vaccine stability can be lost by hydrolysis of the carbohydrate chains, proteolysis, and unfolding of the carrier protein.[128]

Commercial influenza vaccine in the United States is always presented in a single or multidose vial filled with liquid formulation as well as prefilled syringes. Current

influenza vaccines have a listed shelf-life of one year[20], although this is the commercial shelf life; the true shelf-life is likely longer. Typical commercial subunit influenza subunit and live attenuated vaccines have been shown to lose essentially all of their potency after 6 weeks and 2 weeks, respectively, when stored at 37°C.[129, 130]

To increase this shelf-life, many groups are working on developing methods to lyophilize influenza vaccine. Examples excipients included in lyophilized or spray-dried formulations include trehalose[131-135], sucrose, inulin[108, 131, 136], dextran[131-133], and xylose[137]. These work by previously discussed mechanisms of glassy state formation and water replacement interactions. The most successful of these examples demonstrated influenza vaccine stability for up to 12 months at 4°C[133], 6 months at 25°C[133], and 4 months at 40°C.[132] Influenza vaccine MN patches utilize similar stabilization schemes[122, 138-140] as well as methylcellulose[141]. Hirobe *et.al.* also employed sodium hyaluranate to create dissolving MN patches that remained stable for up to six months at 40°C.[142]

2.5 Vaccine characterization techniques

There are a variety of methods to characterize vaccines. Depending on the specific interest, it can be as straightforward as using a vaccine to immunize an animal model to reveal changes in antibody titers or as detailed as a biochemical assessment of protein changes down to the single amino acid residue. The level of investigation is dependent on the stage of vaccine development and the level of detail necessary for the questions being asked.

Arguably the most important technique used to characterize a vaccine is an activity assay. For a human vaccine, human test subjects would be the most ideal model to test the efficacy and activity of a given vaccine, but there are obvious disadvantages of testing vaccines of a significant level of uncertainty on people. Therefore the next step down is to test a vaccine on a suitable animal model. The animal species chosen for testing must be able to respond to vaccination in a way that is correlative to a human's response. This is either having an immune system capable of responding to a specific type of vaccine or having the ability to mimic a human disease.[143] A suitable model for influenza in the murine model.[144] After immunization, the mice produce strain-specific anti-HA

antibodies which can be collected in serum. Antibody titers in serum can be measured by a hemagglutination inhibition assay, as described by the World Health Organization[145], where serum and live virus are incubated together to allow the antibodies to neutralize the viral particles. These neutralized viral particles are then no longer capable of agglomerating added erythrocytes. The amount of erythrocyte agglomeration correlates to the titer of anti-HA antibodies, thus the overall response of each mouse to the immunization.

When an animal model is either not available or too expensive, the next alternative is an *in vitro* activity assay. For live, attenuated vaccines, it is common for cell infectivity to be a marker of vaccine activity. Measles vaccine study utilizes an endpoint tissue infectivity assay to determine the titer of live viral particles that are needed to kill a certain percentage of a cell culture.[146] Samples of live measles vaccine is applied to a monolayer of cells and the active vaccine titer is determined by the percentage of cells killed by measles virus.[146] Development of rubella vaccine involves an immunocolorimetric assay, described by Chen *et.al.[147]*, where a sample of vaccine is applied to a monolayer of cells and allowed to infect these cells, if possible. An antibody specific to a rubella surface protein is then added to bind to rubella particles, allowing for the quantification of live rubella particles in the initial sample.

When there are no live viral particles to measure infectivity, other *in vitro* methods must be used. A common method for measuring the activity of vaccines based around proteins, whether subunit, recombinant, or conjugate, is an enzyme-linked immunosorbent assay, known as an ELISA, as described by Gan and Patel.[148] ELISAs are based on the principle of antibody-antigen binding. There are three different types of ELISA: direct, sandwich, and competitive. In a direct ELISA, a sample containing an antigen of interest, usually a protein, is allowed to bind to a microwell plate. Subsequently, antigen-specific antibody, which is conjugated to an enzyme such as horseradish peroxidase, is added to each well. The conjugated enzyme causes a change in an added substrate such that the change in the substrate is back-correlated with the amount of enzyme, thus the amount of antigen in each well. Other examples of vaccine activity assays. Sandwich ELISAs are very similar to direct ELISAs except that the antigen of interest is not adhered to the well surface, but rather it is bound to an antibody which is adhered to the well surface. This allows for more control over antigen immobilization. The final type of ELISA discussed

here is the competitive ELISA. The key feature here is the competition to bind with antibodies between antigen incubated with antibody and antigen attached to the well surface; higher sample antigen concentration leads to less antibody to bind to surface antigens, thus a lower readout signal.

If the stability of a subunit vaccine is to be studied at a deeper level, there are other, more detailed assays. Many vaccines are suitably sized to be examined by dynamic light scattering (DLS).[149] Whole viral particles can break apart[150] or aggregate[151] which leads to activity loss. This change in particle size distribution should be discernable from DLS measurements of the vaccine sample. Even in the case of influenza subunit vaccine, the HA membrane proteins associate together to form a multimeric rosette which is suitably large for examination by DLS.[152] In addition to using DLS to find the particle size distribution, structural details may be found by transmission electron microscopy.[27] This can help to determine whether there are well-ordered structures or more random aggregation.

Influenza vaccine, based on the antigenicity of HA, is dependent on the structural integrity of the HA protein.[153] One method to monitor general changes to protein structure is intrinsic fluorescence spectroscopy. This intrinsic fluorescence is grounded in the fluorescence of aromatic amino acid residues, such as tryptophan and tyrosine.[154] These residues fluoresce when excited with light in the 270-300 nm wavelength range.[155] The emission spectrum of each is dependent on the local environment of the given residue.[156] Tryptophan is particularly sensitive; the emission spectra is red-shifted as the side group is exposed to a more polar solvent environment.[157] This technique can be useful for exploring tertiary structure changes of proteins.

For investigation of protein secondary structure, one technique is to employ circular dichroism (CD) spectroscopy. The principle effect in CD is the absorbance of variable wavelength polarized light by chiral structures within a protein.[158] Typical protein secondary structure can be described as a combination of alpha helices, beta sheets, and random coils. Each of these motifs have absorb polarized light at signature wavelengths. When a protein is probed with polarized light, an absorbance spectrum is acquired. This spectrum can then be deconvoluted to determine the proportion of each secondary structure the protein is composed of.[159] Therefore, a protein can be analyzed with CD under

various conditions to monitor whether or not there are structural changes as well as provide insights into what those changes are.

CHAPTER III

SPECIFIC AIMS

3.1 Aim 1: Screen microneedle patch formulations and drying conditions to preserve dry influenza vaccine activity

Influenza subunit vaccine can be formulated such that it can retain activity during air drying and during subsequent storage. This aim involved the screening of buffers and formulation excipients to stabilize commercial influenza subunit vaccine during drying and storage at ambient and elevated temperatures. Isolating different categories of formulation excipient allowed for examination of parameters that have an effect on vaccine stability during drying.

3.2 Aim 2: Test stability and immunogenicity of influenza vaccine in microneedle patches

The incorporation of influenza vaccine into suitably formulated microneedle patches causes minimal vaccine activity loss through long-term storage outside of the cold chain, and these patches remain immunogenic. Successful formulations developed in Aim 1 were utilized to produce dissolving polymer microneedle patches with influenza vaccine. These patches were produced and stressed under various conditions they could possibly be exposed to once commercialized. A mouse model was also used to ensure aged patches retain immunogenicity.

3.3 Aim 3: Provide insights into mechanisms of influenza vaccine activity loss during drying

Subunit influenza vaccine loses activity predominantly due to aggregation, which can be inhibited by effective formulations. Influenza vaccine was dried and examined by various analytical techniques to monitor changes at the vaccine particle and protein level. These techniques gave further insights into the stability data from earlier work.

CHAPTER IV

SCREEN MICRONEEDLE PATCH FORMULATIONS AND DRYING CONDITIONS TO PRESERVE DRY INFLUENZA VACCINE ACTIVITY

4.1 Introduction

Influenza is a potentially life-threatening infectious disease that causes up to 5 million cases of severe illness worldwide during seasonal epidemics, while up to 500,000 people die from the disease each year.[160] Annual vaccination against influenza is recommended by the Centers for Disease Control and Prevention (CDC; Atlanta, GA) for everyone 6 months of age and older.[161] The influenza virus is a spherical lipid membrane-enveloped virus. One of its 11 proteins[2] is a spike protein, hemagglutinin (HA), which is the influenza vaccine's primary antigenic protein.[161] This study was conducted using an inactivated purified subunit influenza vaccine where HA and neuraminidase (NA) are in rosette form.

Despite the wide availability of a relatively low-cost vaccine, seasonal influenza vaccination coverage for adults in the United States hovers around just 40%.[162] Two reasons for the low vaccination coverage are patients' needle phobia and inconvenience of travelling to a healthcare setting to receive the vaccine.[163] Currently, the most common route of influenza vaccination is an intramuscular injection of an aqueous suspension of HA. This method has several drawbacks, such as a reliance on the cold-chain for transportation and storage, a possibility of needle-stick injuries, and patients' needle phobia. There are other routes of delivery such as nasal delivery and intradermal injection, which are used in recently approved products and also require cold-chain storage, administration by healthcare personnel and delivery methods that many people find unpleasant.[161]

We and others have proposed the use of microneedle patches for influenza vaccination[68, 164-167] because of the previously demonstrated benefits of less patient

pain[49], improved immune response[167], and possible self-administration[168]. In this way, microneedle patches may enable scenarios where vaccine can be picked up over-the-counter at stores or sent in the mail, so that people can vaccinate themselves and their families at home. An additional feature that would facilitate increased and simplified access to influenza vaccination using a microneedle patch would be reduced reliance on the cold-chain for transportation and storage, thus allowing vaccines to be stockpiled more easily and transported to vaccine recipients. Therefore, the goal of this study is to develop thermostable microneedle patch formulations that can be partially or completely removed from the cold chain.

Much knowledge about preserving vaccines in a dried state comes from the area of lyophilization.[169] The vaccine antigen is typically formulated in the liquid state with various excipients such as buffer salts, bulking agents, and cryoprotectants, and then freeze-dried to form a solid powder. One common approach to vaccine stabilization is to incorporate the vaccine into an amorphous sugar glass. This approach has been taken by a number of investigators to stabilize influenza HA in the dry state by optimizing pH, excipient composition, drying method, and other parameters.[20] Previous work has shown that HA can be stabilized during air drying onto microneedles with sugars such as trehalose and methylcellulose [170, 171]. However, we also showed that the inclusion of surfactant, while useful for coating metal microneedles, leads to matrix crystallization and HA activity loss.[172] While sugars are commonly used stabilizing excipients other compounds, such as amino acids, have been examined as well.[169]

Guided by this previous work, in this study we determined the effect of formulation, drying, and storage conditions on the stability of a licensed sub-unit influenza vaccine during the manufacturing of microneedle patches. Initial screening was carried out by drying vaccine formulations suitable for microneedle patch fabrication and screening them for vaccine stability, which was followed by further formulation composition optimization and long-term stability testing.

4.2 Methods

4.2.1 Vaccine and reagents

Monovalent vaccine stocks were generously provided by Novartis Vaccines and Diagnostics (Cambridge, MA). All studies were conducted using a monovalent stock of B/Brisbane/60/2008 influenza antigen. All excipients were received from Sigma-Aldrich (St. Louis, MO), except for sodium heparin salt (Polysciences, Warrington, PA) and bovine serum albumin (EMD Millipore, Darmstadt, Germany).

4.2.2 Formulations

Vaccine stock solutions were received in phosphate-buffered saline (PBS). Monovalent stock of B/Brisbane/60/2008 received at a concentration of 360 µg/mL was buffer-exchanged using Vivaspin 500, 100 kDa MWCO or Vivaspin 20, 10 kDa MWCO centrifuge filters (Sartorius AG, Göttingen, Germany). Vaccine was concentrated at least 10-fold using the filters, then diluted with the desired buffer. This process was repeated 2-3 times. Finally, the same buffer was used to adjust the vaccine to the appropriate concentration for experiments, which was 60 µg/mL, as determined by ELISA. All buffers were prepared at 300 mosmol/L and adjusted with KOH or HCl to pH 7.0 - 7.4.

To formulate vaccine, dry excipients were weighed and placed into Type 1 glass vials with fused inserts (ChemGlass, Vineland, NJ) or Type 1 glass vials (Wheaton, Millville, NJ), to which buffer-exchanged vaccine stock solution was added. All formulations contained 1% w/v 250 kDa sodium carboxymethyl cellulose (NaCMC). In the study on the effect of surfactant on vaccine stability, each surfactant was added so that the concentration was 50% higher than its critical micelle concentration (see Table 4.1 in Supporting Information). All formulations contained a total stabilizer excipient concentration of either 10% or 15% w/v, where the concentration was split amongst up to four compounds, unless the stabilizer was not soluble to the desired level, in which case the concentration of the stabilizer was its solubility limit at 25°C.

4.2.3 Vaccine stability screening experiments

To increase the throughput of formulation screening, drops of vaccine solution were dried on surfaces representative of coated metal microneedles or dissolving polymer microneedles. To simulate a metal microneedle drying surface, 6 mm-diameter circular “chips” were cut from sheets of Type 301 stainless steel (McMaster, Atlanta, GA). To

simulate the drying surface of dissolving microneedle molds, a thin film of Sylgard 184 (Dow Corning, Midland, MI) was cured, from which 6 mm-diameter circular chips were removed with a hammer punch. Onto these surfaces were placed approximately 2 µL droplets of formulated vaccine solution at a concentration of approximately 100 µg/mL. Droplet weights were recorded on a Sartorius SE2 Ultra Micro Balance (Sartorius AG) for normalization to account for different actual masses administered in each droplet. The vaccine was allowed to dry at 15 – 25% relative humidity without added convection before chips were packaged in aluminum pouches (Oliver-Tolas Healthcare, Grand Rapids, MI) and sealed with an impulse heat-sealer (AIE-300, American International Electric, Industry, CA). All samples were stored with desiccant (Hammond Drierite, Xenia, OH), unless otherwise noted. Chips were then stored for up to 182 days in Model 6020 environmental test chambers maintained at 5°C, 25°C or 40°C (Caron, Marietta, OH). Vaccine activity remaining was calculated by comparing the dried sample to a positive control of similarly prepared vaccine that was not dried to account for any changes during formulation.

4.2.4 ELISA assay

Reconstituted vaccine samples were assayed for hemagglutinin activity using a sandwich enzyme-linked immunosorbent assay (ELISA). Polyclonal, strain-specific antibodies were received from the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA; Silver Spring, MD). Antibodies were conjugated to horseradish peroxidase with a Lightning Link conjugation kit (Innova Biosciences, Cambridge, UK). Unformulated vaccine stock was serially diluted in phosphate-buffered saline with 0.5% Tween-20 (PBST) and used as a reference standard curve. After the prescribed storage time, chips were placed into PBS so that the dried vaccine and excipients were redissolved. This solution was run parallel to the reference standard curve on an Immulon 2HB 96-well microplate (Thermo Scientific, Waltham, MA). The microplate was washed 3 times between each step with PBST with 3% w/v bovine serum albumin. The HRP substrate reaction involved SureBlue Reserve TMB solution (KPL, Gaithersburg, MD), which was stopped with TMB Stop Solution (KPL). The microplate absorbance at 620 nm was read using an iMark plate reader (BioRad,

Hercules, CA). The standard curve was fit to a four-parameter function using the Microplate Manager 6 software (BioRad). HA activity is presented as the percent of HA activity remaining for dried chip samples compared to liquid control solutions.

4.2.5 Statistics

All statistics were calculated using MiniTab software version 17 (MiniTab, State College, PA). All listed averages represent the arithmetic mean of the tested samples. Comparisons within individual samples were performed using an unpaired *t*-test with a significance cutoff of $p<0.05$. For comparisons between three or more samples, a one-way ANOVA was used or the data were fit to a general linear model, depending on the number of parameters varied, to determine significance.

4.3 Results

4.3.1 Drying time/temperature and buffer salts

During microneedle patch manufacturing, a vaccine solution is typically dried on a microneedle surface or in a microneedle mold. To assess vaccine HA activity loss during this drying process, we dried solutions of B/Brisbane/60/2008 influenza vaccine on stainless steel chips to simulate manufacturing of metal microneedles coated with vaccine and on PDMS chips to simulate manufacturing of dissolving polymer microneedles in molds. The vaccine was formulated with NaCMC (to increase viscosity), trehalose (to help stabilize the vaccine during drying, as shown in previous studies[170]) and one of four different buffer salts. Drying was performed at 4°C, 25°C and 40°C for 60, 20 and 7 min, respectively. Each chip was allowed to dry at the designated temperature until the sample was "dry," as determined by no further loss of mass due to water evaporation, i.e., equilibration with the surrounding air with humidity of 15 – 25% (data not shown). Upon drying, each sample was reconstituted and assayed for binding activity by ELISA.

As shown in Figure 4.1, HA activity loss varied approximately from 0% to 50%, depending on vaccine formulation and drying conditions. Drying on stainless steel lead to more activity loss when compared to drying on PDMS (general linear model, $p < 0.001$). Choice of buffer salt also affected activity loss, where PBS performed the worst, and 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and ammonium acetate retained more vaccine activity compared to potassium diphosphate (general linear model, $p < 0.001$ and $p < 0.01$, respectively). Additional analysis showed that different drying temperatures (and thus different drying times, since drying was slower at lower temperature) had a significant effect on vaccine stability also (general linear model, $p < 0.001$), but no statistical difference was found when drying vaccine in ammonium acetate on PDMS at any temperature tested (ANOVA, $p > 0.85$). Based on these findings, we carried out the remaining experiments using vaccine solution buffer-exchanged into ammonium acetate buffer (pH 7.0) and dried on PDMS at room temperature.

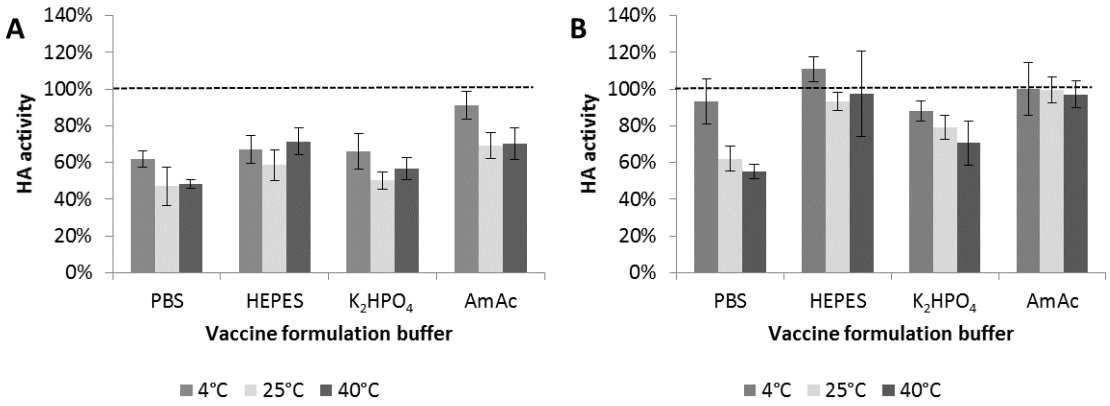

Figure 4.1. Effect of formulation buffer, drying temperature and drying substrate on HA activity after drying influenza trehalose was dried on (A) stainless steel (SS) or (B) polydimethylsiloxane (PDMS) chips at 4°C, 25°C or 40°C and assayed for HA activity after 24 h. PBS = phosphate-buffered saline; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_2HPO_4 = potassium phosphate dibasic; AmAc = ammonium acetate. HA activity is shown as a percentage of concentrated stock vaccine solution after buffer exchanging to the respective vaccine on chips until "dry". Vaccine (B/Brisbane/60/2008) formulated with 15% w/v buffer. Data represent averages of n = 6 replicates, with standard deviation bars shown.

4.3.2 Surfactants

Surfactants are often included in formulations used to coat microneedles.[64] The effects of including surfactants in the vaccine solution formulation were therefore examined by choosing representative surfactants from several categories, including cationic, anionic, zwitterionic and non-ionic. The concentration of each surfactant was

chosen to be 50% higher than its critical micelle concentration (see Table 4.1). First, the effect of preparing liquid vaccine solutions containing surfactants was assessed, which showed that the addition of surfactant to the liquid formulations reduced HA activity in certain cases by up to ~50% (Figure 4.2). Then, the effect of drying these formulations was assessed. We found that there was no significant further loss of HA activity due to the drying in most cases (Figure 4.2).

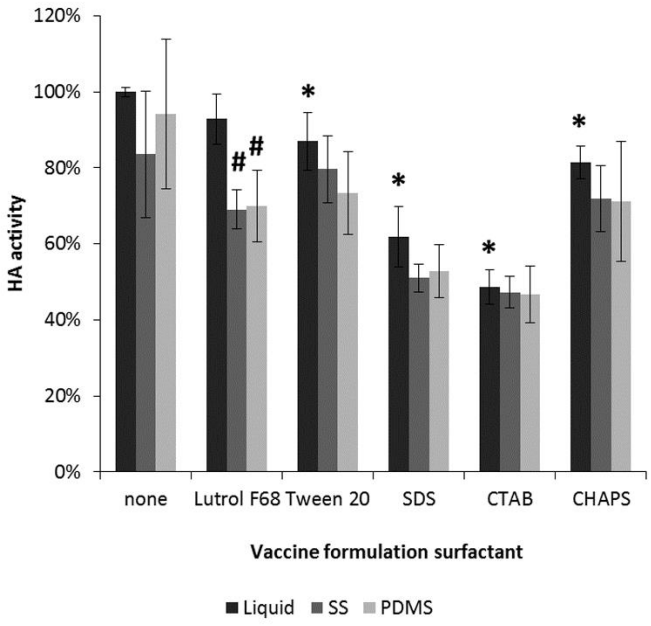

Figure 4.2. Effect of surfactant on HA activity after drying influenza vaccine on PDMS or stainless steel chips at 25°C until "dry". Vaccine (B/Brisbane/60/2008) was formulated with 15% w/v trehalose in ammonium acetate buffer. Asterisk (*) represents significant loss of HA activity in the liquid formulation containing surfactant compared to vaccine stock solution (Student's t-test, $p < 0.05$). Hash (#) represents significant loss of HA activity in the dried formulation compared to the liquid formulation containing surfactant (Student's t-test, $p < 0.05$). HA activity is shown as a percentage of activity of concentrated stock vaccine solution with the corresponding surfactant. Data represent averages of n = 6 replicates, with standard deviation bars shown. Surfactants and their critical micelle concentrations can be found in Table 4.1.

Table 4.1. Surfactants and their critical micelle concentrations from Figure 4.2.[173]

Surfactant	critical micelle concentration (mM)
Lutrol F68	0.04
Tween 20	0.06
SDS (sodium dodecyl sulfate)	8
CTAB (cetrimonium bromide)	1
CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)	6

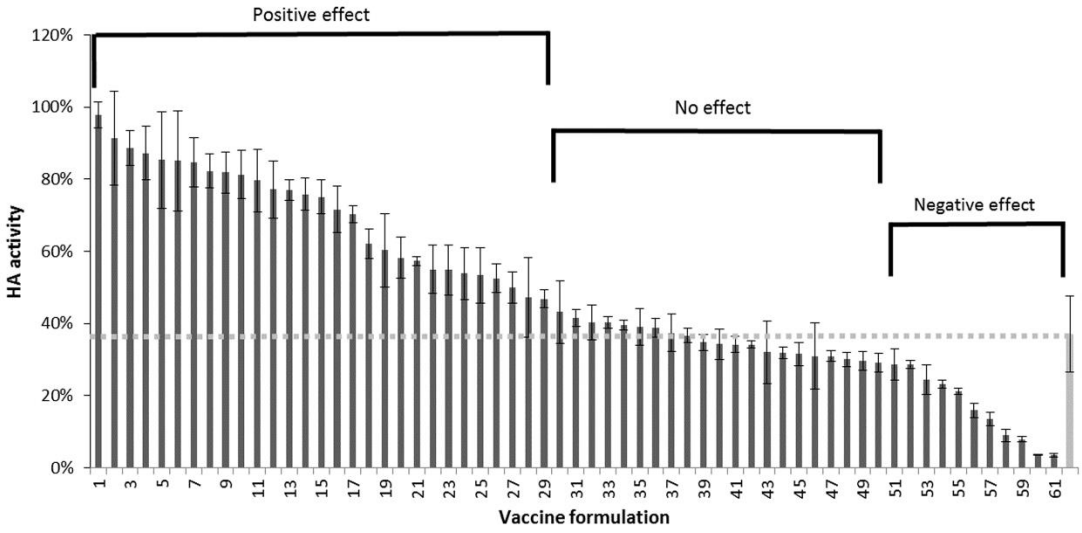

Figure 4.3. Effect of stabilizing excipients on HA activity after drying influenza vaccine on PDMS chips and storing with desiccant for 24 h at room temperature (25°C). Vaccine (B/Brisbane/60/2008) was formulated with individual excipients at a concentration of 15% in ammonium acetate buffer (See Table 4.2 for key to excipient list). Excipients had a positive effect (i.e., HA activity was significantly greater than the dried, unformulated vaccine indicated by the gray bar on the far right; Student's t-test $p < 0.05$), no significant effect ($p > 0.05$) or a negative effect ($p < 0.05$). HA activity is shown as a percentage of concentrated stock vaccine solution. Data represent averages of n = 6 replicates, with standard deviation bars shown (Student's t-test, $p < 0.05$).

Table 4.2. Key to vaccine formulation excipients found in Figure 4.3.

Formulation #	Stabilizer	Activity remaining	Formulation #	Stabilizer	Activity remaining
1	maltodextrin 4	98%	32	lysine	40%
2	arginine	91%	33	sodium phosphate	40%
3	trehalose	89%	34	valine	40%
4	maltose	87%	35	threonine	39%
5	histidine	85%	36	galactose	39%
6	calcium heptagluconate	85%	37	alanine	37%
7	maltodextrin 13	85%	38	methionine	37%
8	sucrose	82%	39	proline	35%
9	glucose	82%	40	mannitol	34%
10	heparin	81%	41	human serum albumin 2	34%
11	raffinose	80%	42	glutamine	34%
12	myo-inositol	77%	43	human serum albumin 1	32%
13	lactose	77%	44	tryptophan	32%
14	sorbitol	76%	45	tyrosine	32%
15	arabitol	75%	46	serine	31%
16	fructose	72%	47	bovine serum albumin	31%
17	γ-cyclodextrin	70%	48	creatine	30%
18	potassium gluconate	62%	49	leucine	30%
19	adonitol	60%	50	potassium sulfate	29%
20	xylitol	58%	51	sodium succinate	29%
21	sodium thiosulfate	57%	52	isoleucine	29%
22	asparagine	55%	53	ethyl lactate	24%
23	2-hydroxypropyl-β-cyclodextrin	55%	54	phenylalanine	23%
24	Tris	54%	55	arabinose	21%
25	sodium citrate	53%	56	potassium phosphate	16%
26	dulcitol	53%	57	xylose	14%
27	potassium citrate	50%	58	potassium sulfite	9%
28	ovalbumin	47%	59	cysteine	8%
29	glycine	47%	60	glycerol	4%
30	methyl glucoside	43%	61	sodium thioglycolate	4%
31	β-cyclodextrin	42%			

Although surfactants did not generally affect HA stability during drying, the surfactants did generally reduce HA activity in the solution state before drying and previous studies have shown that surfactants can damage influenza vaccine during extended storage, possibly due to direct effects on the vaccine, such as solubilization of the viral membrane[174], or indirect effects on the vaccine, such as increased crystallization of formulation components[172]. For this reason, further experiments were performed without surfactant in the formulation.

4.3.3 Stabilizer screening

Our next step was to screen excipients that further stabilize HA activity during drying. Formulated vaccine was dried on PDMS chips and stored for 24 hours at room temperature with desiccant. As shown in Figure 4.3, there was a broad range of effects, spanning from almost complete loss of activity to almost complete retention of activity. These HA activity levels were compared to that of vaccine that was dried in the absence of

excipients (i.e., the bar on the far right of the graph) and classified into three categories: compounds that stabilized the vaccine (i.e., greater stability than the unformulated control, Student's t-test, $p < 0.05$), compounds that had no significant effect ($p > 0.05$), and compounds that destabilized the vaccine ($p < 0.05$). Most of the compounds that stabilized HA activity were carbohydrates along with a few amino acids.

To identify formulations that stabilize HA not only during drying, but also during subsequent storage, dried vaccine samples were stored for 1 week at 40°C as a second level of screening. Because there can be improved effects when multiple stabilizers are used together[175], we selected the best stabilizers from the initial screen and assessed their effect alone or in pairwise combination with each other. This second, more-stringent screen identified individual stabilizers that continued to be effective and identified stabilizer combinations that were effective as well (Figure 4.4). In most cases, stabilizer combinations were no better than at least one of the individual stabilizers, except for two combinations (Figure 4.4).

Based on the one-week screening study, vaccine formulations were prepared using the top five stabilizers, as well as each two-stabilizer combination of these excipients, dried and stored at 40°C for up to one month. While most formulations were stable up to one week, most of them also lost substantial HA activity at one month (Figure 4.5). Three combinations, however, had no significant activity loss during the one month of storage. These combinations were trehalose/sucrose, sucrose/arginine, and arginine/heptagluconate.

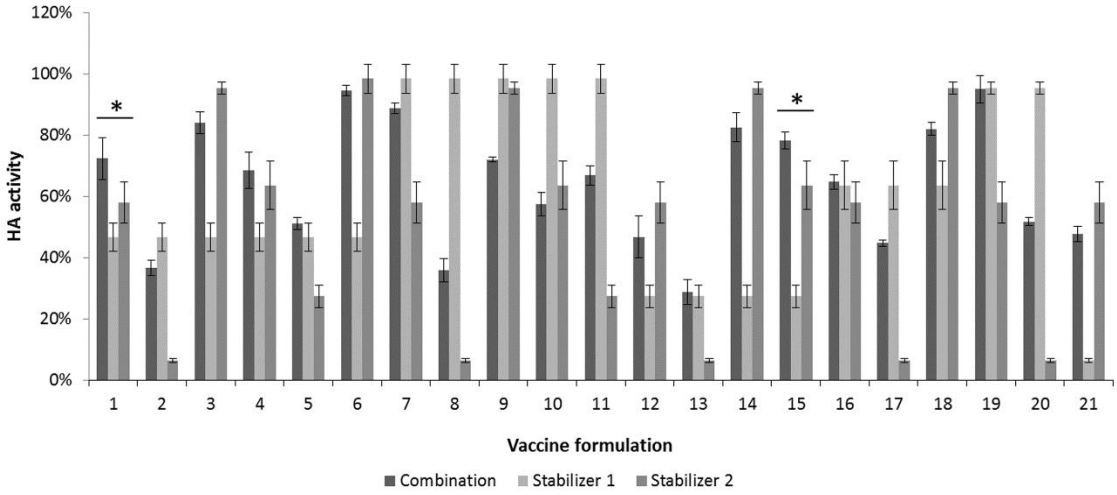

Figure 4.4. Effect of combinations of stabilizing excipients on HA activity after drying influenza vaccine on PDMS chips and storing with desiccant for one week at 40°C. Vaccine (B/Brisbane/60/2008) was formulated with either one or two stabilizing excipients at a total stabilizer concentration of 15% w/v in ammonium acetate buffer (See Table 4.3 for key to excipient list). In each set of bars, the first bar shows HA activity for the combination formulation, the second bar shows HA activity for formulation with just the first stabilizing excipient and the third bar shows HA activity for formulation with just the second stabilizing excipient. Asterisk (*) indicates combination-excipient formulations with significantly higher HA activity compared to either associated individual-excipient formulations (Student's t-test, $p < 0.05$). HA activity is shown as a percentage of concentrated stock vaccine solution. Data represent averages of n = 5 replicates, with standard deviation bars shown.

Table 4.3. Key to vaccine formulation excipients found in Figure 4.4.

Formulation #	Stabilizer 1	Stabilizer 2
1	maltodextrin	lactose
2	maltodextrin	sodium thiosulfate
3	maltodextrin	trehalose
4	maltodextrin	sodium citrate
5	maltodextrin	sorbitol
6	maltodextrin	arginine
7	arginine	lactose
8	arginine	sodium thiosulfate
9	arginine	trehalose
10	arginine	sodium citrate
11	arginine	sorbitol
12	sorbitol	lactose
13	sorbitol	sodium thiosulfate
14	sorbitol	trehalose
15	sorbitol	sodium citrate
16	sodium	lactose
17	sodium	sodium thiosulfate
18	sodium	trehalose
19	trehalose	lactose
20	trehalose	sodium thiosulfate
21	sodium thiosulfate	lactose

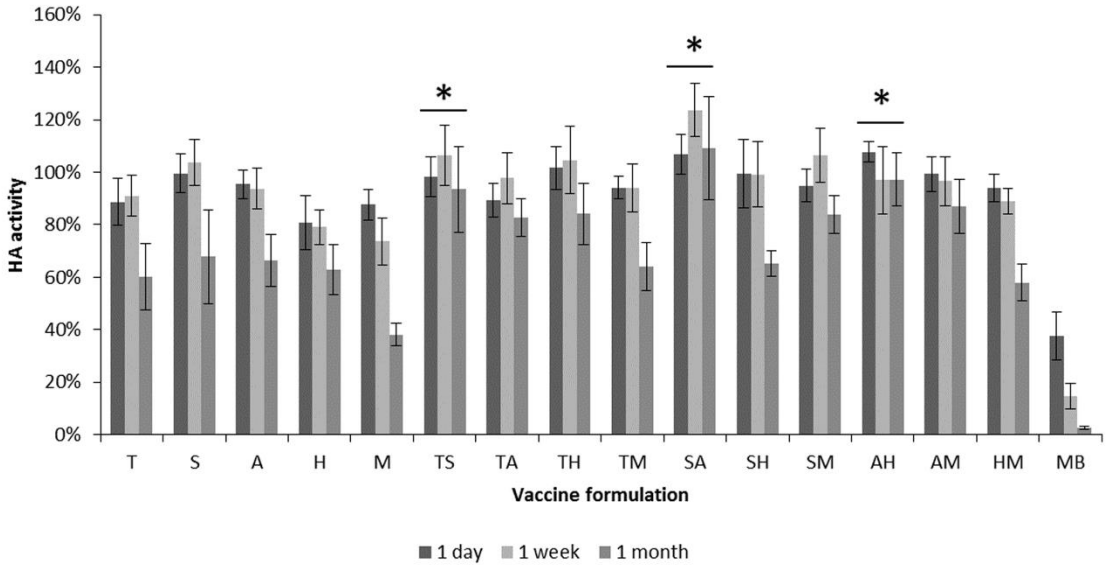

Figure 4.5. Effect of stabilizing excipients on HA activity after drying influenza vaccine on PDMS chips and storing with desiccant for up to one month at 40°C. Vaccine (B/Brisbane/60/2008) was formulated with individual or pair-wise combinations of stabilizing excipients at a total stabilizer concentration of 15% w/v in ammonium acetate buffer. HA activity is shown as a percentage of concentrated stock vaccine solution. T= trehalose, S = sucrose, A = arginine, H = calcium

heptagluconate, M = maltodextrin 13, MB = monobulk. Asterisk (*) indicates no statistically different HA activity amongst the three time points (Student's t-test, $p < 0.05$). Bars represent averages of n = 5 replicates; with standard deviation bars shown.

4.3.4 Long-term stability of chips

Using the optimal vaccine formulations identified in the one-month screen, we carried out a six-month screen using those excipients in pairwise combination at formulation ratios of 90:10, 50:50, and 10:90, as well as combinations of three excipients and all four excipients combined in equal amounts (Figure 4.6). Unformulated vaccine lost nearly all of its activity within the first month of testing. All the formulations tested in this study were generally stable for the eighteen months of study without an effect of stabilizer ratio. At the eighteen-month time point, none of the formulations showed significant loss in HA activity relative to day 0 (Student's t-test, $p < 0.05$).

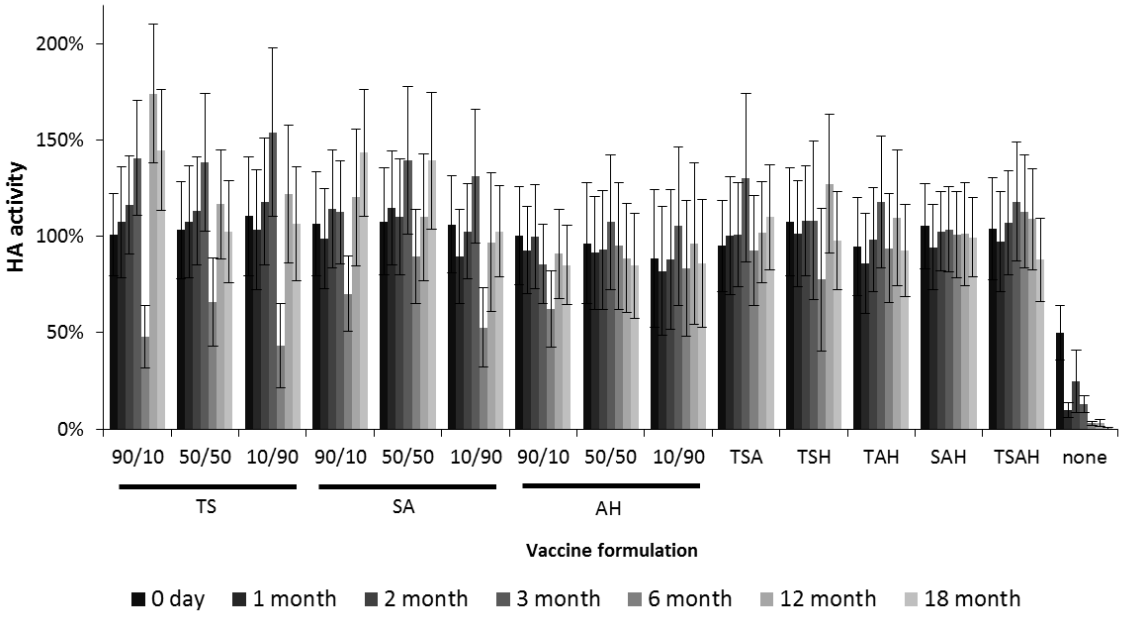

Figure 4.6, Effect of stabilizing excipient formulation on hemagglutinin (HA) activity after drying influenza vaccine on PDMS chips and storing with desiccant for up to twelve months at 40°C. Vaccine (B/Brisbane/60/2008) was formulated with combinations of two, three, or four stabilizing excipients at a total stabilizer concentration of 10% w/v in the vaccine casting solution. For the two-stabilizer combinations, the concentration ratio is shown, where the first number corresponds to the relative content of the first excipient listed below it on the x axis. The three-way and four-way formulation had equal amounts of each excipient. T = trehalose, S = sucrose, A = arginine, H = sodium heptagluconate. HA activity is expressed as a

percentage of HA activity in the liquid casting solution. Data represent averages of n = 12 replicates, with standard deviation bars shown.

4.3.5 Packaging humidity

To examine the effect of humidity during storage, several single-excipient formulations were dried on PDMS chips and stored at 40°C for up to 16 weeks in aluminum pouches with either desiccant, to maintain a humidity approaching zero, or conditioned silica gel pouches, to maintain a relative humidity of 70%. As seen in Figure 4.7, desiccated samples were generally stable, whereas samples stored at elevated humidity rapidly lost HA activity.

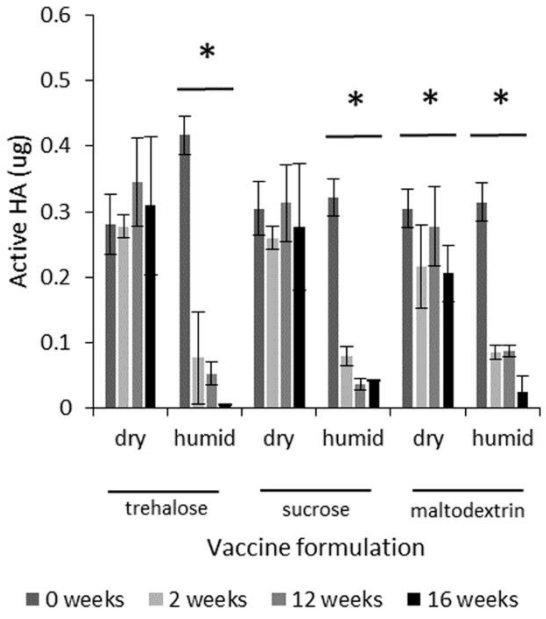

Figure 4.7. Effect of humidity on HA activity after drying influenza vaccine on PDMS chips and storing with desiccant for up to 16 weeks at 40°C. Vaccine (A/Brisbane/59/2007) was formulated with trehalose, sucrose or maltodextrin at a concentration of 10% w/v in ammonium acetate buffer. Chips were stored with either desiccant (dry) or silica gel that had been conditioned to maintain 70% relative humidity (humid). Asterisk (*) indicates a significant change in HA activity over time (ANOVA, $p < 0.05$). HA activity is shown as the mass of active HA. Data represent averages of n=9 replicates, with standard deviation bars shown.

4.4 Discussion

This study seeks to develop formulations towards a thermostable microneedle patch for influenza vaccination. Through a series of screening and optimization studies to develop formulation compositions and manufacturing parameters that enhance thermostability of the HA antigen in influenza vaccine, we developed microneedle patch formulations that protected the influenza vaccine antigen during eighteen months of storage at an elevated temperature.

In the study design, we expected two timescales of HA activity loss. The first is the initial drying phase where the liquid formulation is allowed to air-dry over the course of minutes to hours, thereby stressing the antigen as water is removed. The second timescale occurs over days to months, as the dried antigen and its matrix can slowly change during storage. The study plan therefore included initial screening of formulations and manufacturing conditions that protected the vaccine during the initial drying phase, and then built on those results to further develop formulations that protected the vaccine during storage.

The optimization was carried out on "chips" that simulated the manufacturing process without the need to go through the more laborious process of making actual microneedle patches. Advantages of this approach included ease of operation, control of antigen loading, and reduced amount of antigen required per test. The PDMS chips were representative of PDMS molds used during manufacturing of dissolving microneedles[176] and the stainless steel chips were representative of the metal microneedles used during manufacturing of coated microneedles. Based on the first set of experiments, we identified drying on PDMS at room temperature using ammonium acetate buffer as our baseline operating condition. We chose PDMS as the drying substrate because HA activity was more stable on PDMS than on stainless steel, and because we are primarily interested in making dissolving microneedle patches that generate no sharps waste, which are manufactured using PDMS molds. We chose room temperature, because there was not a significant effect of temperature on HA activity when ammonium acetate was the buffer, and room temperature is simpler to implement than cooled or heated drying, and is faster than drying at low temperature. Although HEPES buffer performed similarly well, we chose ammonium acetate buffer because it maintained HA activity during drying and, as a volatile buffer, it will partially evaporate during drying, thereby leaving fewer

crystals.[177] PBS performed the worst, perhaps because of pH drops when dibasic phosphate precipitates before monobasic phosphate.[83]

Surfactants were found to destabilize HA activity, especially in liquid formulation before drying. Previous work has further shown that surfactants can cause crystallization in the microneedle coating matrix during storage and cause damage to HA activity.[172] We therefore chose not to include surfactants in our formulation, since they are not typically needed when molding dissolving microneedles, although they are often used when coating metal microneedles.[66]

Using these baseline operating conditions, we screened a large number of excipients selected to stabilize HA activity during drying and subsequent storage. Candidate excipients were selected from the literature and past data from our laboratory showing stabilization of HA and other proteins and vaccines, included conventionally used stabilizers as well as other compounds not well known for their stabilizing properties. We found that approximately 45% of the excipients tested provided significant stabilizing effects, almost 20% destabilized HA and the remainder had no significant effect when compared to drying the vaccine with no additional excipients. The large majority of successful stabilizers were carbohydrates, including small saccharides, polyols, and long-chain polysaccharides. Two notable exceptions were the stabilizing amino acids, arginine and histidine.

We next tested combinations of the best-performing stabilizers, some of which performed better than their individual excipient components. The best-performing formulations generally contained arginine and calcium heptagluconate either in pairwise combination or as components of formulations containing additional excipients as well, such as both trehalose and sucrose. Considering the stabilizer ratio of the formulation, we generally found among a set of two excipient combinations, a composition of 50:50 retained the most antigen activity.

The development of a thermostable influenza vaccine patch could facilitate greater influenza vaccination coverage during both seasonal and pandemic influenza scenarios. The cold chain contributes substantially to the cost of storing and transporting vaccine doses.[178, 179] In addition, thermostability is important to our long-term vision of a self-administered microneedle patch for influenza vaccination,[168] where vaccine could be

stored on non-refrigerated shelves in the pharmacy and could be mailed to patients at home without ice packs.

4.5 Conclusion

This study had the objective to develop a thermostable microneedle patch for influenza vaccination. Initial studies focusing on maintaining stability during the drying of influenza vaccine associated with microneedle patch manufacturing determined that HA activity was higher after drying on PDMS than on stainless steel, was only sometimes significantly affected by drying temperature at 4°C, 25°C or 40°C, was more effectively stabilized by ammonium acetate and HEPES buffers than phosphate-based buffers, and was destabilized by addition of surfactants. Subsequent studies assessed the ability of 60 excipients to stabilize HA activity during drying and subsequent storage at elevated temperature. The best excipients, and excipient combinations, were then identified as those that maintained HA activity for up to eighteen months at 40°C when stored with desiccant. The best performing stabilizer combination was arginine/heptagluconate. This work will be utilized to further develop a dissolving microneedle patch that can remain independent of the cold chain. We conclude that the formulations developed here may enable development of a thermostable microneedle patch for influenza vaccination to improve future coverage during seasonal and pandemic vaccination.

4.6 Acknowledgements

We thank Novartis Vaccines and Diagnostics for generously providing monovalent influenza vaccine stock. This work was supported in part by the National Institutes of Health. We thank Polo Gaputan and Miraj Desai for their work on this project. The work was carried out in the Center for Drug Design, Development and Delivery, and the Institute for Bioengineering and Bioscience at the Georgia Institute of Technology. Matthew Mistilis, Andreas Bommarius and Mark Prausnitz are inventors of patent(s) that have been or may be licensed to companies developing microneedle-based products, and Mark Prausnitz is a paid advisor to companies developing microneedle-based products and is a founder/shareholder of companies developing microneedle-based products. This potential

conflict of interest has been disclosed and is overseen by Georgia Tech and Emory University.

CHAPTER V

TEST STABILITY AND IMMUNOGENICITY OF INFLUENZA VACCINE IN MICRONEEDLE PATCHES

5.1 Introduction

Influenza is estimated to cause up to five million illnesses and five hundred thousand deaths each year.[160] Annual seasonal influenza vaccination is recommended by the Centers for Disease Control and Prevention[161], and more than 148 million people received the vaccine in the United States during the 2014-15 influenza season.[180] As a result, there is significant cost, complex logistics and vaccine spoilage due to the need to distribute and store the vaccine in the cold-chain .[181] Elevated temperatures as well as exposure to freeze-thaw cycles have been shown to adversely affect the stability of seasonal influenza vaccine.[130]

One method to maintain the activity of vaccines, or proteins in general, is lyophilization. For many proteins, preservation in a dry state enables storage for longer time or without refrigeration.[182] In a dry state, protein mobility is greatly reduced,[183] and there is less water available for many decomposition reactions.[184] Lyophilized biopharmaceuticals must still be reconstituted with an appropriate diluent and administered via a hypodermic needle and syringe. All current influenza vaccines are stored and delivered in the liquid state and also require the use of the cold-chain. The cold-chain can cost hundreds of million dollars per vaccine program[181], thus an influenza vaccine stable at room temperature could be of significant financial benefit.

We and others are developing microneedle patches for vaccination against influenza and other diseases.[65, 70, 140, 171, 185, 186] Microneedle patches contain an array of sub-millimeter projections composed primarily of water-soluble sugars and polymers that encapsulate a payload of drug or vaccine.[176] Since these patches hold the vaccine in a dry state, once produced, microneedle patches can maintain stability similar to lyophilized vaccine. To achieve this stable state, formulation excipients must be optimized to maintain vaccine activity during manufacturing and subsequent storage.

During manufacturing, a vaccine and excipient mixture is cast and dried into molds and allowed to dry, which can damage the vaccine. There has been work to develop formulation strategies to stabilize influenza vaccine during microneedle fabrication. For example, silk[187] and trehalose[139] have been incorporated into microneedle formulations to stabilize influenza vaccine during drying. Aside from stability enhancement, microneedles could increase the acceptance of vaccine administration[168] and improve the immune response in recipients[69, 188] .

Formulation excipients that function as lyoprotectants of proteins are often small sugars.[111, 179, 189, 190] There are thought to be two different, yet possibly simultaneous, mechanisms by which sugars and polyols maintain protein stability in a dried state.[179, 189] One is the formation of an amorphous sugar glass phase with such a high viscosity that molecular mobility is severely restricted.[191] This restricted mobility decreases kinetics of possible degradation pathways, both chemical and physical.[183] The other mechanism is the replacement of removed water molecules' hydrostatic interactions by hydroxyl groups from the sugars,[192] which is needed because water molecules form a stabilizing hydration shell around proteins in solution.[193] Polysaccharides, such as inulin[194], and amino acids, such as arginine[119], have also been used to stabilize proteins, due also to advantageous excipient/protein interactions and the creation of a favorable dry matrix.

In a previous study, we screened 61 different formulation excipients and some of their combinations to find three pairwise excipient combinations that were especially promising at stabilizing a trivalent subunit influenza vaccine: trehalose/sucrose, sucrose/arginine, and arginine/heptagluconate.[195] In this study, we further examined these formulations for stability at 25°C and 40°C over periods up to 18 months and during environmental stresses such as exposure to 60°C for four months, multiple freeze-thaw cycles, and electron beam irradiation.

5.2 Methods

5.2.1 Vaccine and reagents

Monovalent vaccine stocks were generously provided by Novartis Vaccines and Diagnostics (Cambridge, MA). Studies with monovalent vaccine dried on chips and in microneedle patches stored for eighteen months utilized B/Brisbane/60/2008 influenza antigen, while for studies testing stability at 60°C, freeze conditions, and irradiation utilized A/Brisbane/10/2010 (H1N1) influenza antigen. Studies with trivalent vaccine utilized B/Brisbane/60/2008, A/Brisbane/59/2007 (H1N1) and A/Victoria/210/2009 (H3N2) influenza antigens. All excipients were received from Sigma-Aldrich (St. Louis, MO).

5.2.2 Vaccine formulation

Vaccine stock solutions were received in phosphate buffered saline (PBS). To buffer exchange into ammonium acetate buffer, monovalent stock solutions of the desired strain was concentrated at least 10-fold using Vivaspin 500, Vivaspin 20, or Vivaspin 2 centrifuge filters with molecular weight cutoffs of 100, 10, and 3 kDa, respectively (Sartorius AG, Göttingen, Germany) and then diluted with 150 mM ammonium acetate buffer. This process was repeated 2-3 times. Finally, the ammonium acetate buffer was used to adjust the vaccine to the appropriate concentration for experiments.

To formulate vaccine for casting onto molds, dry excipients were weighed and placed into Type 1 glass vials (Wheaton, Millville, NJ), to which buffer-exchanged vaccine stock solution was added. All formulations contained 1% w/v 250 kDa sodium carboxymethyl cellulose (NaCMC). All formulations contained a total stabilizing excipient concentration of 10% w/v, where the concentration was split amongst up to four compounds.

5.2.3 Vaccine stability testing in microneedle patches

Microneedle patches were prepared using two different casting solutions. First, a PDMS mold in the shape of the microneedle patch was prepared, using methods described previously.[196] The vaccine casting solution consisted of influenza vaccine, 1% w/v NaCMC, and 10% w/v of stabilizer in 150 mM ammonium acetate buffer. The matrix casting solution consisted of polyvinyl alcohol (PVA), sucrose, and water in a mass ratio of 8:6:15. To make microneedle patches, vaccine casting solution was placed on a mold to

fill the microneedle cavities with the aid of vacuum. Excess vaccine solution was removed from the mold surface with a flat blade. The vaccine casting solution was then allowed to dry into the tips of the microneedle mold cavities under vacuum. Next, matrix casting solution was placed on the mold and allowed to dry under vacuum, thereby forming a complete microneedle patch. Each patch was then stored in a desiccator at room temperature (~21 - 25°C) for two days before demolding with Scotch tape (3M, St. Paul, MN).

Demolded patches were packaged with desiccant in aluminum pouches and stored at a given temperature (i.e., 4°C, 25°C or 40°C) in stability chambers. For patches subjected to freeze-thaw cycles, each cycle consisted of two hours at -20°C followed by two hours at 4°C. Patches subjected to electron-beam sterilization were irradiated using a 10 MeV (20 kW) system at room temperature for a total exposure of 10 or 20 kGy. The nonirradiated controls were subjected to the same travel and storage conditions as the test samples.

5.2.4 ELISA assay

Reconstituted vaccine samples were assayed for hemagglutinin activity using a sandwich enzyme-linked immunosorbent assay (ELISA). Polyclonal, strain-specific antibodies were received from the Center for Biologics Evaluation and Research of the Food and Drug Administration (Silver Spring, MD). Antibodies were conjugated to horseradish peroxidase with a Lightning Link conjugation kit (Innova Biosciences, Cambridge, UK). Unformulated vaccine stock was serially diluted in PBS with 0.5% Tween-20 (PBST) and used to generate a reference standard curve. After the prescribed storage time, chips and microneedle patches were placed into PBST so that the dried vaccine and excipients were redissolved. This solution was run parallel to the reference standard curve on an Immulon 2HB 96-well microplate (Thermo Scientific, Waltham, MA). The microplate was washed three times between each step with PBST containing 3% w/v bovine serum albumin. The horseradish peroxidase (HRP) substrate reaction involved SureBlue Reserve TMB (3,3’,5,5’-tetramethylbenzidine) solution (KPL, Gaithersburg, MD), which was stopped with TMB Stop Solution (KPL). The microplate absorbance at 620 nm was read using an iMark plate reader (BioRad, Hercules, CA). The standard curve was fit to a four-parameter function using the Microplate Manager 6 software (BioRad).

5.2.5 Immunization of mice

Immunization of mice

Groups of female BALB/c mice (n=8 per group), all 6-8 weeks of age, were immunized via either an intradermal (ID) injection at the base of the tail or by application of a microneedle patch to the back. In the first study, animals were vaccinated with 3.8 µg, 2.8 µg, and 3.8 µg of H1N1, H3N2, and B strains, respectively, administered either by ID injection of fresh stock vaccine or using microneedle patches stored at 25 °C for 13 months. In the second study, animals were vaccinated with 1 µg, 0.6 µg, and 0.6 µg of H1N1, H3N2, and B strains, respectively, by ID injection of fresh stock vaccine, by ID injection of vaccine reconstituted from microneedle patches stored at 25 °C for 13 months or using freshly made microneedle patches. All patches were made using arginine and heptagluconate at a mass ratio of 50:50 as the stabilizers in the vaccine casting solution.

Hair was removed one day prior to immunization using electric clippers and a depilatory cream (Nair; Ewing, NJ). Patches were applied by pressing the patch against a pinch of hair-free skin using the thumb and forefinger for 1 min. Adhesive on the patch backing held the patch in place for a further 20 min to ensure microneedle dissolution in the skin. Blood was drawn via the jugular vein at days 0, 14, and 28. Whole blood was then spun down in serum separation tubes (Becton Dickinson, Franklin Lakes, NJ) to collect the serum.

5.2.6 Hemagglutination inhibition titers

Production of influenza-specific antibodies was determined by hemagglutination inhibition (HAI) assay. Serum was treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) overnight at 37°C. Samples were then incubated at 56°C for 30 min before being incubated overnight with packed red blood cells at 4°C. After centrifugation, supernatant was collected, serially diluted in PBS, and mixed with separate strain-specific influenza viruses for 30 min. Finally, 0.5% chicken red blood cells was added. The reciprocal of highest dilution titer that prevented hemagglutination was read as the HAI titer.

5.2.7 Electron beam irradiation

Electron beam irradiation was conducted by Synergy Health (Lima, OH) using a 10MeV (20 kW) system, irradiated at 10 or 20 kGy. The irradiations were conducted at ambient temperatures. The nonirradiated controls were subjected to the same travel and storage conditions as the test samples.

5.2.8 Statistics

All statistics were calculated using Excel 2013 software (Microsoft, Redmond, WA). Reported averages represent the arithmetic mean of the tested samples, except for HAI titers, which are presented as the geometric mean titer for each group. Seropositivity was considered to be an HAI titer greater than or equal to 40. Comparisons between individual samples were performed using an unpaired t-test with a significance cutoff of $p<0.05$.

5.3 Results

5.3.1 Stability of influenza vaccine patches over time at room temperature

Guided by the screening results generated on chips showing that all of the two-, three- and four-way combinations of stabilizers effectively stabilized the vaccine, we made microneedle patches containing monovalent vaccine using the three optimal two-way combinations and tested their stability during storage at 25°C for 18 months. These patches were made by first casting a vaccine solution containing the antigen and stabilizing excipients and, after drying, casting a matrix solution containing sucrose and PVA to fill any remaining space not occupied by the dried vaccine solution and to form the patch backing. Among the microneedle patches with two-way combinations of stabilizing excipients, only the arginine/heptagluconate formulation exhibited no significant loss of vaccine activity after 18 months (Fig. 5.1, Student's *t*-test, $p > 0.05$).

Using just the individual stabilizers showed that formulation with any of the saccharides resulted in no significant loss of vaccine activity after 18 months (Fig. 5.1, Student's *t*-test, $p > 0.05$), but there was a significant decrease in vaccine activity at 18 months when arginine was used as the only stabilizer (Fig. 5.1, Student's *t*-test, $p = 0.03$). The unformulated vaccine also had no loss of activity after 18 months (Fig. 5.1, Student's

t-test, $p > 0.05$). However, the patches using unformulated vaccine and vaccine formulated with the single stabilizers sucrose, arginine or heptagluconate had significant losses in activity during fabrication on Day 0 by comparison with vaccine activity in patches formulated with arginine-heptagluconate as a positive control (Fig. 5.1, Student's t-test, $p < 0.05$). This is probably due to differences in vaccine stabilization associated with vaccine drying during patch fabrication. Comparison of these findings in microneedle patches to the previous findings on chips indicates that the excipients in the matrix casting solution used to make the microneedle patch backing provided additional stabilization.

Based on these findings with monovalent patches, we assessed the stability of trivalent patches using the three two-way combinations of stabilizers. There was no significant change in vaccine activity for any of the three vaccine strains using the arginine/heptagluconate formulation after storage for 18 months at 25°C, as well as the H1N1 strain when formulated with trehalose/sucrose (Fig. 5.2, Student's *t*-test, $p > 0.05$). There was also no noticeable change in the appearance of the patches after storage compared to freshly prepared patches (Fig. 5.3a-b). These findings further indicate the superiority of the arginine/heptagluconate formulation as a stabilizer.

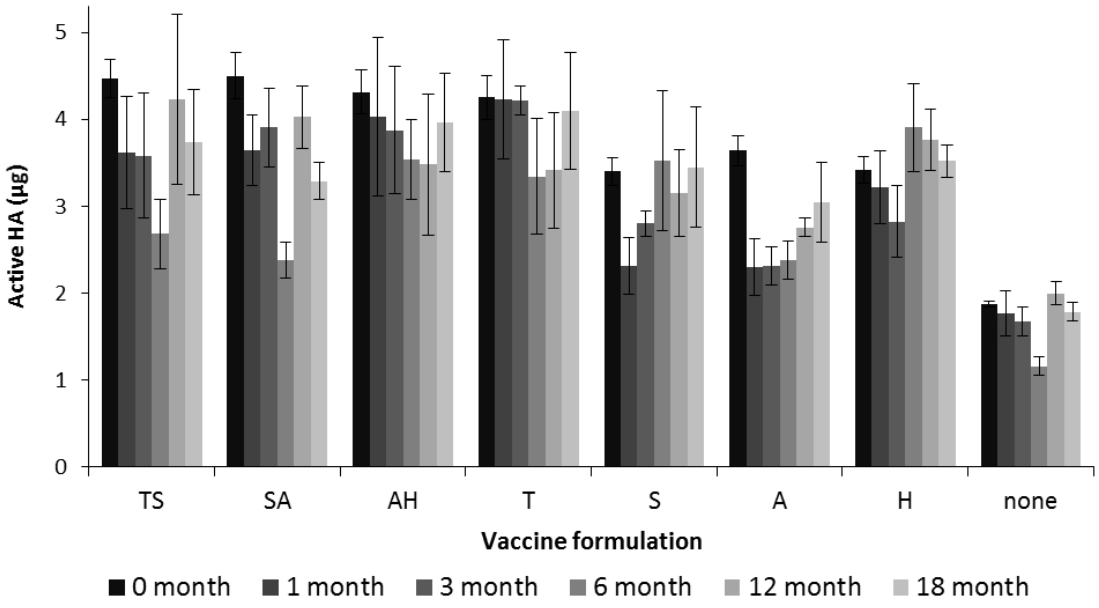

Figure 5.1. Effect of formulation on hemagglutinin (HA) activity after fabricating microneedle patches and storing with desiccant for up to 18 months at 25°C. Monovalent microneedle patches were fabricated with B/Brisbane/60/2008 influenza vaccine formulated with stabilizing excipients at a total concentration of 10% w/v in the vaccine casting solution. The two-stabilizer combinations contained equal amounts of each excipient. T = trehalose, S = sucrose, A = arginine, H = sodium heptagluconate. HA activity is shown as the mass of active HA in each patch. Data represent averages of n = 6 replicates, with standard deviation bars shown.

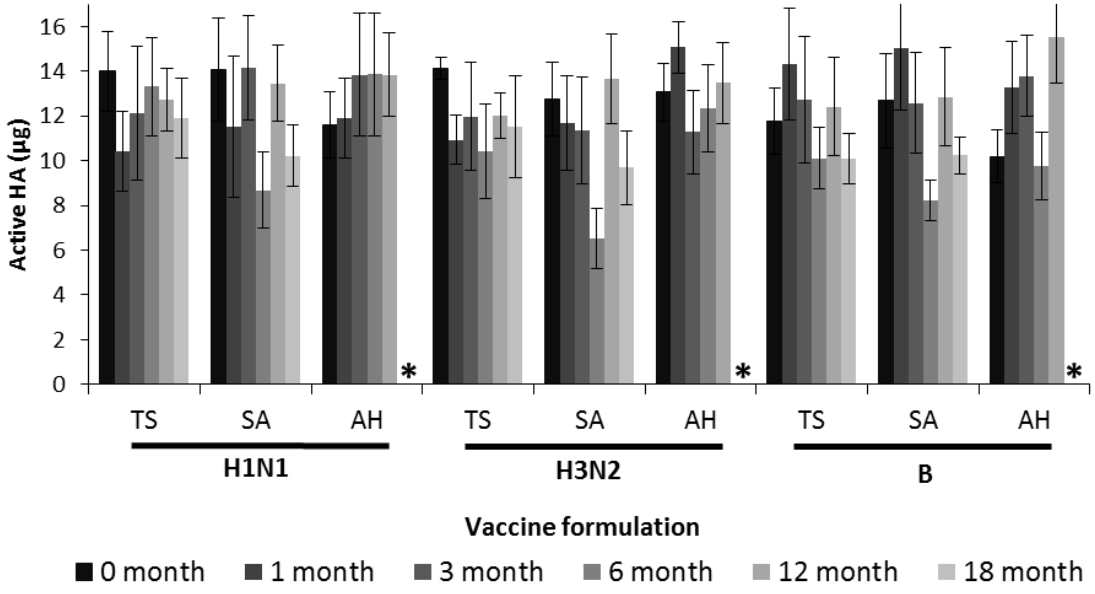

Figure 5.2. Effect of formulation on hemagglutinin (HA) activity after fabricating microneedle patches and storing with desiccant for up to 18 months at 25°C. Trivalent microneedle patches were fabricated with A/Brisbane/59/2007 (H1N1), A/Victoria/210/2009 (H3N2) and B/Brisbane/60/2008 (B) influenza vaccine formulated with two-way combinations of stabilizing excipients at a ratio of 50:50 with a total stabilizer concentration of 10% in the vaccine casting solution. T = trehalose, S = sucrose, A = arginine, H = sodium heptagluconate, * = data not available. Data represent averages of n = 6 replicates, with standard deviation bars shown.

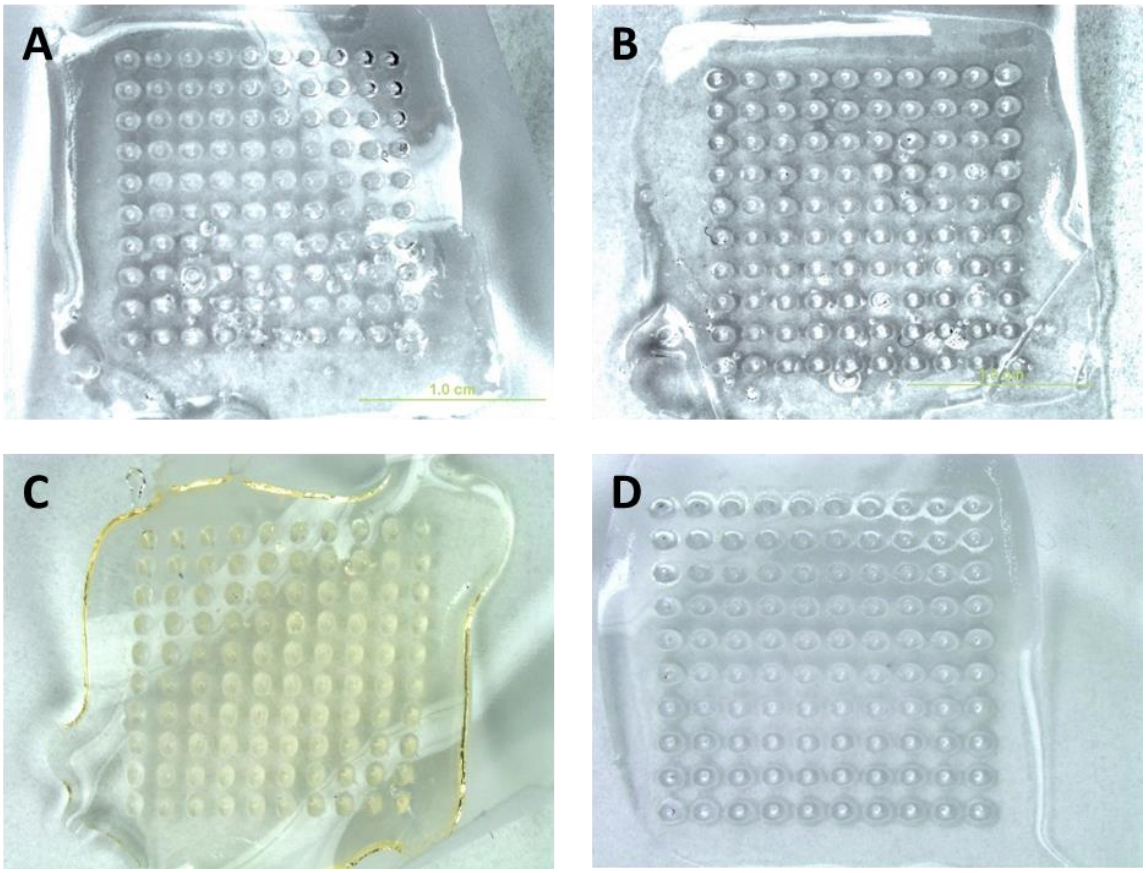

Figure 5.3. Images of representative microneedle patches (A) immediately after production, (B) after storage at 25C for 18 months, (C) after storage at 60C for two months, and (D) after five freeze-thaw cycles.

5.3.2 Immunogenicity of stored influenza vaccine patches

To determine if the *in vitro* vaccine stability of microneedle patches correlated with *in vivo* immunogenicity, mice were immunized using microneedle patches containing trivalent influenza vaccine that had been stored for 13 months at 25°C and compared to ID injection of fresh vaccine. There was no significant difference in immune response to the H1N1 influenza strain between the mice immunized with a stored microneedle patch and by an ID injection (Fig 5.4a). Immune response to the H3N2 strain was higher for stored microneedle patches than ID injection (Fig. 5.4b). For both strains, all mice were seropositive after vaccination with stored microneedle patches, but only 50% – 70% were seropositive after ID vaccination (Fig 5.4c – 5.4d). However, immune responses to the B

strain of influenza could not be measured due to lack of access to the virus needed for the assay.

The superior immune response of stored microneedle patch vaccination compared to ID vaccination may at first be surprising. However, comparison of fresh microneedle patch vaccination to ID vaccination similarly showed improved immune response after microneedle patch vaccination (Fig. 5.5), indicating that the superior response was not associated with storage, but was instead associated with the microneedle patch method of vaccination, which is consistent with prior literature[69, 197]. Moreover, ID injection of vaccine reconstituted from stored microneedle patches generated immune responses not significantly different from ID injection of fresh vaccine (Fig. 5.5), further suggesting that stored vaccine was as immunogenic as fresh vaccine and that improved immunogenicity was associated with microneedle patch administration.

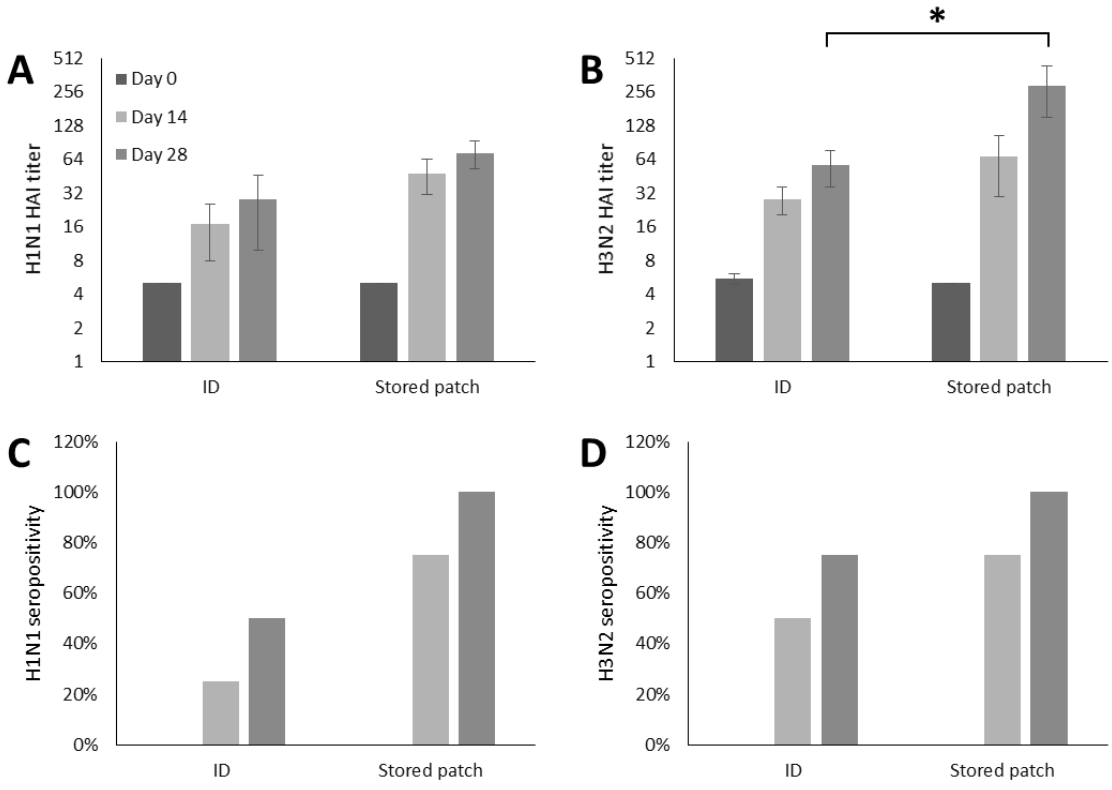

Figure 5.4. Immune responses of BALB/c mice immunized with trivalent influenza vaccine. Strain-specific hemagglutination inhibition (HAI) titers are represented as geometric means of n = 8 mice, with ± standard error of mean bars shown for (A) H1N1 and (B) H3N2 influenza vaccination. Asterisk (*) indicates a significant

difference in antibody titers (Student's t-test, $p < 0.05$). Seropositivity data for (C) H1N1 and (D) H3N2 influenza vaccination are represented as the percentage of each experimental group that achieved an antibody titer of ≥40. Trivalent microneedle patches were fabricated with A/Brisbane/59/2007 (H1N1), A/Victoria/210/2009 (H3N2) and B/Brisbane/60/2008 (B) influenza vaccine formulated with a two-way combination of arginine and heptagluconate each at a concentration of 5% w/v in the vaccine casting solution ID = intradermal vaccination, Stored patch = vaccination with a microneedle patch after storage at 40°C with desiccant for 13 months. Data from vaccination with B/Brisbane/60/2008 are not available due to technical limitations.

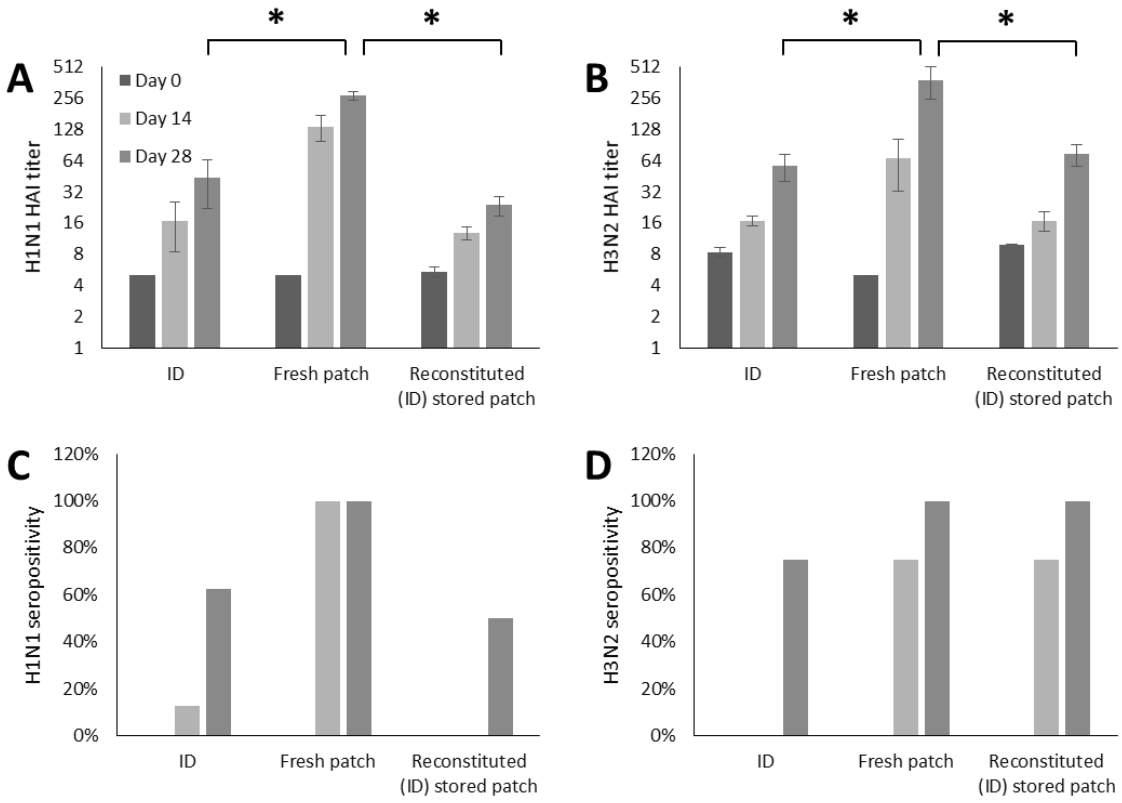

Figure 5.5. Immune responses of BALB/c mice immunized with trivalent influenza vaccine. Strain-specific hemagglutination inhibition (HAI) titers are represented as geometric means of n = 8 mice, with ± standard error of mean bars shown for (A) H1N1 and (B) H3N2 influenza vaccination. Asterisk (*) indicates a significant difference in antibody titers (Student's t-test, $p < 0.05$). Seropositivity data for (C) H1N1 and (D) H3N2 influenza vaccination are represented as the percentage of each experimental group that achieved an antibody titer of ≥40. Trivalent microneedle patches were fabricated with A/Brisbane/59/2007 (H1N1), A/Victoria/210/2009 (H3N2) and B/Brisbane/60/2008 (B) influenza vaccine formulated with a two-way combination of arginine and heptagluconate each at a concentration of 5% w/v in the vaccine casting solution ID = intradermal vaccination, Fresh patch = vaccination with a microneedle patch, Reconstituted (ID) stored patch = intradermal vaccination using

a reconstituted microneedle patch after storage at 40°C with desiccant for 13 months. Data from vaccination with B/Brisbane/60/2008 are not available due to technical limitations.

5.3.3 Stability of influenza vaccine patches at elevated temperature

While microneedle patches stored at 25°C simulates vaccine storage in a climate-controlled pharmacy or warehouse, we wanted to determine if microneedle patches remained stable at higher temperatures. Microneedle patches containing monovalent influenza vaccine were therefore stored at 60°C for four months. There was no significant loss of vaccine activity for these patches as a function of time (Fig. 5.6, ANOVA, $p > 0.05$) or in comparison to patches stored in parallel at 25°C (Fig. 5.6, ANOVA, $p > 0.05$). Visual inspection of the patches indicated minor discoloration of the patch after storage at 60°C, suggesting that patch excipients may have been affected (Fig. 5.3c).

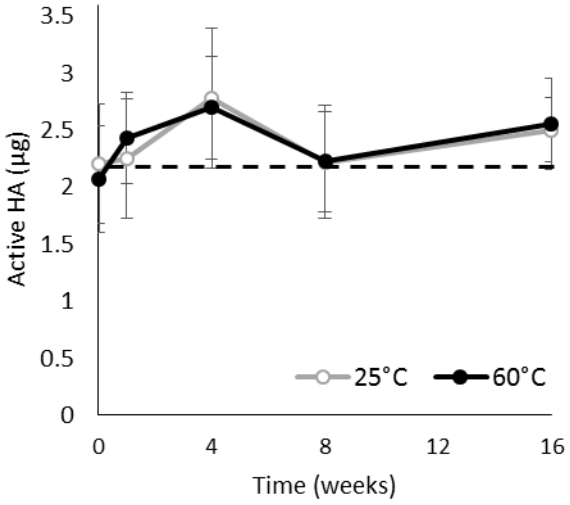

Figure 5.6. Stability of influenza vaccine in microneedle patches stored 60°C with desiccant for up to four months compared to patches stored at 25°C under identical conditions. Monovalent microneedle patches were fabricated with A/Brisbane/10/2010 influenza vaccine formulated with a two-way combination of arginine and heptagluconate each at a concentration of 5% w/v in the vaccine casting solution. Data from patches stored at 60°C are shown, as well as control patches which were stored at 25°C and tested simultaneously. HA activity is shown as the mass of active HA in each patch. Data represent averages of n = 6 replicates, with standard deviation bars shown.

5.3.4 Stability of influenza vaccine patches after multiple freeze-thaw cycles

Vaccines can be exposed to freeze-thaw cycles if stored without environmental controls or during poorly-controlled refrigeration. To address such conditions, microneedle patches containing monovalent influenza vaccine were subjected to five freeze-thaw cycles, alternating between -20°C and 4°C, over the course of 24 h, after which there was no significant change in vaccine activity compared to patches maintained at 25°C (Fig. 5.7, Student's t-test, $p = 0.95$). Patches maintained at 4°C or at -80°C also had no significantly loss of activity compared to patches stored at 25°C (Fig. 5.7, Student's t-test, $p > 0.05$). Interestingly, unformulated liquid vaccine subjected to identical freeze-thaw cycles also showed no loss in vaccine activity (Fig. 5.7, Student's t-test, $p = 0.88$). The appearance of patches after the freeze-thaw cycles showed no apparent differences from fresh patches (Fig. 5.3d).

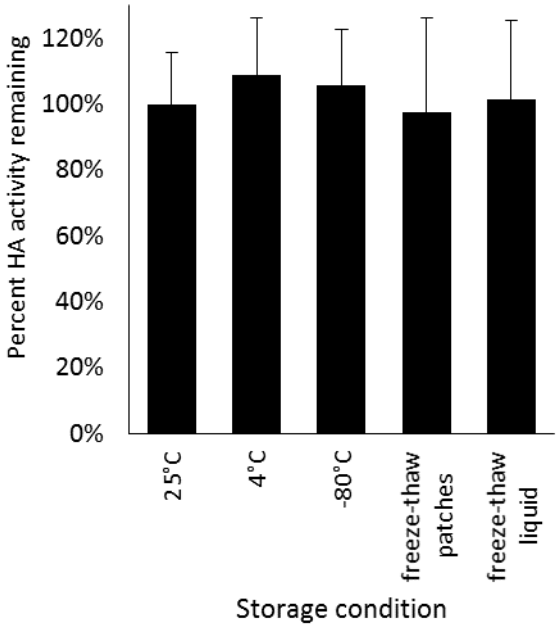

Figure 5.7. Stability of influenza vaccine in microneedle patches subjected to five freeze-thaw cycles. Monovalent microneedle patches were fabricated with A/Brisbane/10/2010 influenza vaccine formulated with a two-way combination of arginine and heptagluconate each at a concentration of 5% w/v in the vaccine casting

solution. All samples were stored for a total of 24 hours. Freeze-thawed samples were subjected to five freeze-thaw cycles of 2 h each at 4°C followed by 2 h at -20°C. Patches stored at 25°C served as the positive control. Patches were also stored at 4°C and -80°C for comparison. Finally, liquid vaccine was subjected to the same freeze-thaw cycles. Data represent averages of n = 6 replicates, with standard deviation bars shown.

5.3.5 Stability of influenza vaccine patches after electron-beam sterilization

Terminal sterilization of microneedle patches could provide cost savings relative to aseptic manufacturing. Microneedle patches containing monovalent influenza vaccine were therefore subjected to electron beam sterilization at two different radiation doses. Compared to untreated patches, there was no significant loss of vaccine activity at either radiation dose (Fig. 5.8, Student's t-test, $p > 0.05$). Patch appearance after irradiation showed no apparent changes from fresh patches (image not available).

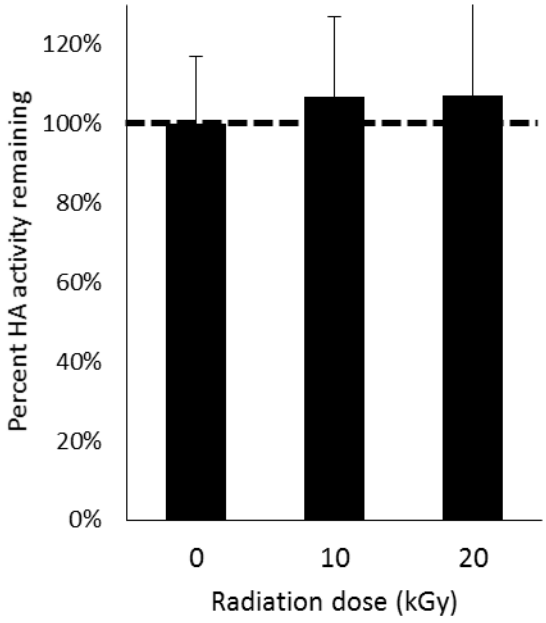

Figure 5.8. Stability of influenza vaccine in microneedle patches exposed to electron beam irradiation. Monovalent microneedle patches were fabricated with A/Brisbane/10/2010 influenza vaccine formulated with a two-way combination of arginine and heptagluconate each at a concentration of 5% w/v in the vaccine casting solution. HA activity is shown as the mass of active HA in each patch. Data represent averages of n = 6 replicates, with standard deviation bars shown.

5.4 Discussion

This work builds upon previous work that included a screen of microneedle formulation excipients to stabilize influenza vaccine.[195] The stability of dried vaccine on chips as well as in patches stored for up to 18 months at 25°C was reported in that work. The work reported here shows further evidence of stability when influenza vaccine was incorporated into dissolving microneedle patches and maintained activity after storage at 25°C for at least 18 months, 60°C for at least four months, five freeze-thaw cycles and electron beam irradiation. The ability to withstand this wide array of stressful conditions suggests that a properly formulated microneedle patch could enable influenza vaccine to be removed from the cold chain. Resistance to electron beam irradiation suggests that terminal sterilization may be possible, as opposed to the more costly scenario of aseptic manufacturing, which could enable significant cost savings.

Influenza vaccine stability, however, requires proper formulation. It is well known that trehalose and sucrose are good lyoprotectants[172, 191, 194, 198, 199], and arginine, a basic amino acid, has been shown to both inhibit aggregation[119] and dry into an amorphous state[200]. Calcium heptagluconate has received limited attention as a stabilizer, but has been shown to stabilize hemoglobin in the liquid state,[201] which was the reason we included this excipient in our initial stabilizer screen. A possible reason for the success of multiple-stabilizer formulations relative to formulations using single stabilizers (i.e., in chips, without the presence of additional formulation excipients used in the complete microneedle patch production) is the inhibition of crystallization of one excipient by the other excipient(s).[202]

Some microneedle patches were made without a stabilizer in the first mold fill. These patches showed significantly less active vaccine after fabrication, but did not have additional losses once dried and placed in storage. This indicates that the stabilizers included in the vaccine casting solution were needed predominately for stability during the fabrication process. Once the patches were completed, dried, and stored, the sucrose and PVA that compromise the bulk of the microneedles and backing provides sufficient stabilization to maintain vaccine activity during long-term storage. These findings of monovalent vaccine stability also held true for microneedle patches containing trivalent

influenza vaccine. A dose comparable to the full human dose in clinical use was incorporated into these patches, which suggests that these findings can be readily translated into microneedle patches used to administer seasonal influenza vaccine to people.

As further tests of influenza vaccine stability in microneedle patches, patches utilizing the arginine/heptagluconate formulation were subjected to very high temperature, freeze-thaw cycling, and electron beam irradiation. Over a period of four months, vaccine in microneedle patches lost no significant activity when stored at 60°C. While we do not envision patches needing to withstand such a high temperatures for so long, it suggests that the microneedle patch could survive most any expected elevated thermal exposure that a seasonal influenza vaccine might experience during its one-year shelf life. The excellent stability during freeze-thaw cycling suggests the ability of influenza vaccine in microneedle patches to survive freezing that could occur due to handling errors or faulty cold-chain equipment. The ability of microneedle patches to preserve vaccine activity at both -80^{o}C and after five consecutive freeze-thaw cycles could be associated with the relatively low moisture content of a few percent (data not shown). This small amount of water may be bound water that does not form ice crystals[203] or it may be phase-separated or otherwise in poor contact with the vaccine antigen, so that they do not interact.

Of relevance to the anticipated future use of microneedle patches for clinical influenza vaccination, maintenance of vaccine activity after exposure to sterilizing irradiation by electron beam suggests that terminal sterilization of microneedle patches could be use. This would avoid the high cost of aseptic manufacturing and therefore represent a significant cost savings. These findings, however, should be tempered by the fact that there is evidence that damaging effects of electron beam irradiation may not be apparent until after storage[204] (which we did not study) and that the sterilization cycle used in this study is expected to be suitable for microneedle patch sterilization, but sterility of these patches was not verified.

The primary test of vaccine activity in this work was performed by ELISA, which is a reasonable measure of vaccine stability, but is not the gold standard method, which is single radial immunodiffusion (SRID).[205] We did not use SRID in this study because it is a complex assay that is difficult to operate reproducibly and is relatively low throughput. Instead, we verified our *in vitro* findings with *in vivo* studies of immunogenicity in mice.

When compared to ID injection of a comparable vaccine dose, stored microneedle patches stored for more than one year at 25°C elicited an equal or stronger immune response after vaccination. This increase in antibody response when compared to ID delivery was seen in this study when using freshly made microneedles patch and has been noted before in the literature.[69, 197] To remove the effect of delivery route, stored microneedles were dissolved in water and injected ID. Mice receiving either fresh vaccine or stored vaccine delivered ID generated indistinguishable antibody responses, showing that the vaccine in the stored patches had retained its immunological activity.

When considered in the context of the broader microneedle patch literature, we believe this study further suggests that influenza vaccination using microneedle patches could be a valuable improvement over current vaccination practice. Previous studies indicate that vaccination using microneedle patches provides improved immune responses, probably due to targeting of the vaccine to the skin.[206] In addition, microneedle patches are small, which facilitates storage, distribution and storage, and because they dissolve in the skin, microneedles generate no biohazardous sharps waste.[65, 70, 140, 171, 185, 186] In part because they are painless and can be self-administered, human subjects have indicated a strong preference for microneedle patches over hypodermic needle injections.[168] This study adds one more reason why influenza vaccination could benefit from microneedle patch administration; the remarkable thermostability demonstrated here suggests that influenza vaccine could be taken out of the cold chain to decrease costs, decrease vaccine spoilage and increase convenience of vaccination.

5.5 Conclusion

This study shows that influenza vaccine formulated into microneedle patches remained stable after storage for 18 months at 25°C, storage for four months at 60°C, exposure to five freeze-thaw cycles and irradiation during electron-beam sterilization. Most stability measurements were made by assessing activity of monovalent vaccine by ELISA; additional studies using trivalent influenza vaccine assessed by ELISA and measuring antibody responses in mice gave similar results. A microneedle patch for influenza vaccination with this high level of stability could enable vaccine storage and distribution

outside the cold chain to simplify and reduce costs of annual influenza vaccination campaigns and vaccine stockpiling for emergencies.

5.6 Acknowledgments

We thank Novartis for generously providing monovalent influenza vaccine stock. This work was supported in part by the National Institutes of Health. We thank Polo Gaputan and Miraj Desai for their work on this project. We also thank Dr. Richard Compans and Dr. Ioanna Skountzou for helping to plan the animal study. We thank Jessica Joyce for assistance carrying out the animal study as well as, including Stein Esser, for assistance in analyzing the serum for antibody titers. The work was carried out in the Center for Drug Design, Development and Delivery, and the Institute for Bioengineering and Bioscience at the Georgia Institute of Technology. Matthew Mistilis, Andreas Bommarius and Mark Prausnitz are inventors of patent(s) that have been or may be licensed to companies developing microneedle-based products, and Mark Prausnitz is a paid advisor to companies developing microneedle-based products and is a founder/shareholder of companies developing microneedle-based products. This potential conflict of interest has been disclosed and is overseen by Georgia Tech and Emory University.

CHAPTER VI

PROVIDE INSIGHTS INTO MECHANISMS OF INFLUENZA VACCINE ACTIVITY LOSS DURING DRYING

6.1 Introduction

It is estimated that up to 500,000 deaths occur each year worldwide due to influenza infections.[160] In addition to the human cost, there is also a large economic impact. In the United States alone, it is estimated the economy bears a burden between $70-167 billion each year.[207] The US Centers for Disease Control and Prevention recommend that everyone above the age of six months receive a seasonal influenza vaccination.[161] The current influenza vaccine is delivered primarily via hypodermic needle and syringe, although there is currently an alternative vaccine delivered intranasally.[208] Vaccine delivered by either of these routes must be stored and transported within the cold-chain.[209] The ability to remove the cold-chain from consideration for a vaccine would lower vaccination campaign costs and lower the possibility of vaccine wastage or the delivery of non-immunogenic doses.

A novel vaccine delivery system under development and currently undergoing clinical trials[210] is the microneedle patch. This technology utilizes an array of sub-millimeter projections to deliver drugs directly into the skin.[59] Of particular interest to this work is the variety of microneedle patches that employs water soluble microneedles. These microneedles are composed of water-soluble excipients, such as sugars and polymers. The purpose of these excipients is to provide mechanical strength for insertion into skin, to enable quick dissolution after insertion, and to provide a stable environment for the drug, which is encapsulated inside of the microneedle itself.[176] This latter is of particular interest for the ability to remove these microneedle patches from the cold-chain.

A common method of biopharmaceutical stabilization is lyophilization.[211] This process freezes a drug solution then removes the vast majority of water by sublimation and heating.[212] The removal of water, along with proper excipient selection, can help to stabilize the drug in a highly immobile state with fewer deleterious interactions.[189] Due

to the nature of microneedles as a solid dosage form, there is an opportunity for dry protein stabilization. This work is focused on elucidating the mechanism of influenza vaccine activity loss during drying as part of the microneedle manufacturing process and how certain formulations affect its stability.

There are several different forms of influenza vaccine, but the most common form is the subunit vaccine. Subunit vaccines are composed primarily of membrane proteins of inactivated and broken down viral particles. In the case of the influenza vaccine, the principle antigenic protein of interest is hemagglutinin (HA).[131] HA is a ~225 kDa (i.e. 3 x 75 kDa) homotrimer that is responsible for sialic acid binding on the surface of a target cell as well as membrane fusion to that cell, facilitating the introduction of viral cargo into the cell.[5] HA is synthesized as a single polypeptide precursor, HA_0, which then non-covalently associates into trimers. This non-active, fusion-incompetent assembly is cleaved during translation to the viral membrane to form a native, mature HA protein composed of two subunits, HA_1 and HA_2, linked by a single disulfide bond.[213] HA membrane fusion follows a conformational change to a fusogenic HA state brought on by low endosomal pH after endocytosis.[18]

There has been previous work to stabilize influenza vaccine in various states such as liquid[214, 215], lyophilized[131, 216], spray (freeze)dried[20], and incorporated into microneedle patches[140]. This work, while generalizable to a degree, is focused on dissolvable microneedle patches where a vaccine/excipient solution is added to a microneedle patch mold and allowed to air dry. We have found that the changes to the HA protein upon drying are primarily related to tertiary structure changes as opposed to secondary structure changes, which can be predominately prevented with the choice of suitable formulation components.

6.2 Methods

6.2.1 Vaccine and reagents

Monovalent vaccine stocks were generously provided by Novartis Vaccines and Diagnostics (Cambridge, MA). Influenza vaccines used were strains A/Christchurch/16/2010(H1N1), A/Texas/50/2012(H3N2), and A/Victoria/361/2011(H3N2). All three strains were used for circular dichroism and

intrinsic fluorescence experiments; A/Christchurch/16/2010(H1N1) and A/Texas/50/2012(H3N2) were used for dynamic light scattering and transmission electron microscopy experiments; A/Victoria/361/2011(H3N2) was used for proteolysis experiments. All excipients were purchased from Sigma-Aldrich (St. Louis, MO). Vaccine used in circular dichroism studies was buffer exchanged into 10 mM phosphate buffer with 20 mM sodium fluoride (KPNF), pH 7.3, overnight at 4°C using ThermoFisher Slide-A-Lyzer dialysis cassettes with a 10 kDa molecular weight cutoff (Waltham, MA). Vaccine used for all other studies was buffer exchanged twice at 6°C into 150 mM ammonium acetate, pH 7.0, with Sartorius (Göttingen, Germany) Vivaspin 500 centrifuge filters with a 30 kDa molecular weight cutoff.

When dried vaccine samples were needed, the vaccine samples were dried for 24-48 hours in Costar prelubricated microcentrifuge tubes at room temperature with desiccant and a vacuum of 85 kPa. When applicable, a solution of 30% excipient was mixed with the vaccine sample before drying.

6.2.2 Dynamic light scattering (DLS)

All DLS measurements were acquired on a Malvern Instruments Zetasizer Nano ZS90 (Malvern, UK) in Eppendorf UVette cuvettes (Hamburg, Germany). Data was acquired with ten 30 second measurements per sample. Data shown represent three samples per condition which were averaged with Malvern Zetasizer Software v7.11.

6.2.3 Transmission electron microscopy (TEM)

5 µl of influenza vaccine in suspension was placed onto 400 mesh carbon-coated copper grids that had been made hydrophilic by glow discharge. After 2 minutes, grids with the protein suspension were rinsed by briefly touching the sample side with three drops of distilled water. The residual water on the grids was then removed by wicking the edge of the grids with a piece of filter paper. For negative staining, 5 µl of 1% aqueous uranyl acetate was applied onto the grids immediately after water removal, and then wicked dry as described above after 10 seconds. Grids were left to completely dry prior to imaging on a JEOL JEM-1400 transmission electron microscope (Tokyo, Japan).

6.2.4 Proteolysis digestion

After reconstitution of dried vaccine and preparation of liquid vaccine samples, 1 mg/mL proteinase K was added at a 1:1 v/v ratio of HA to proteinase K. This mixture was allowed to incubate at 25°C for one hour. After incubation with enzyme, 100% trichloroacetic acid was added at a 1:4 volume ratio. This mixture was incubated at 4°C followed by centrifugation to pellet the protein in each tube. This pellet was then rinsed at least three times with acetone. After allowing the residual acetone to dry, pellets were rehydrated. Samples were incubated for 10 minutes at 90°C with sodium dodecyl sulfate sample buffer lacking a reducing agent before being separated by electrophoresis using 170 V across a 12% acrylamide gel for up to one hour and stained with Coomassie Brilliant Blue.

6.2.5 Circular dichroism (CD)

Heated samples were diluted to their final volume with KPNF and placed in a 75°C water bath for three hours. Liquid samples were diluted to their final volume with KPNF and scanned without further processing. All CD measurements were acquired on a Jasco J-815 CD spectrometer (Easton, MD). Liquid samples were placed in Jasco 1 mm path length Q-quartz cuvettes and scanned at wavelengths from 250 nm to 190 nm at a 200 nm/min and 10 accumulations per sample. Post-analysis consisted of smoothing by the Savitzky-Golay method and a convolution width of 9, scaling each curve at 220 nm, and averaging the three samples for each condition.

6.2.6 Intrinsic protein fluorescence

Dried vaccine samples were dried for 24 hours in Corning round bottom black microwell plates (Corning Inc., Corning, NY) at room temperature with desiccant and a vacuum of 85 kPa. When applicable, a solution of 30% excipient was mixed with the vaccine sample before drying. Dried samples were then rehydrated so that the fluorescence emission spectra could be acquired. Samples were excited at 280 nm and the emission was measured from 300-400 nm on a BioTek Synergy MX (Winooski, VT). To mitigate the

effect of noise during scanning, spectrum peak determinations were calculated by fitting a third-order polynomial to the raw fluorescence intensity from 300-350 nm.

6.2.7 Statistics

All statistics were calculated using Excel 2013 software (Microsoft, Redmond, WA). All listed averages represent the arithmetic mean of the tested samples. Comparisons between individual samples were performed using an unpaired t-test with a significance cutoff of $p < 0.05$.

6.3 Results

6.3.1 Electron microscopy

Samples of vaccine were examined with a transmission electron microscope (TEM) to help clarify the results of DLS studies. TEM images showed an abundance of multimeric rosettes of HA molecules with a characteristic size of 30 – 40 nm, as is typical of subunit influenza vaccine[217] (Fig 6.1a). In addition, larger structures were also noted (Fig 6.1b). They appeared to be vesicle-like assemblies of HA molecules. They were typically in the size range of 100-200 nm in diameter, and the frequency of occurrence did not appear to be a function of sample preparation.

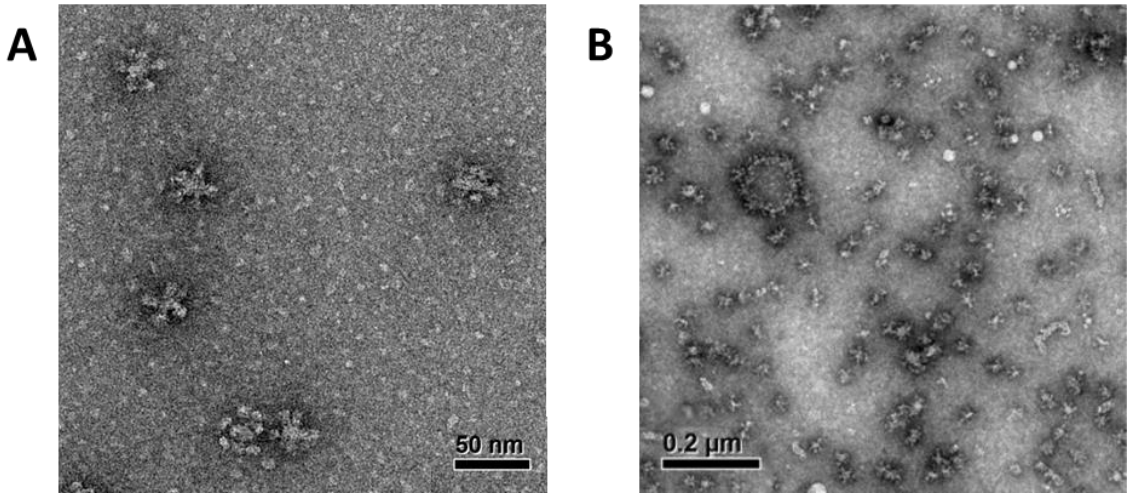

Figure 6.1. Transmission light microscopy images of A/Texas/50/2012 at two magnifications to show (A) multimeric rosettes and (B) multimeric rosettes alongside larger protein assemblies.

6.3.2 Dynamic light scattering

The distribution of hydrodynamic radii of vaccine components was determined using dynamic light scattering (DLS). Typical intensity distribution curves of liquid control vaccine reveal two unique peaks, the first at 20-40 nm diameter, which is interpreted as hemagglutinin (HA) rosettes[218], and the second at 100-300 nm diameter, which is interpreted as larger assemblies of HA (Fig 6.2a). The number distribution curve (Fig 6.2b) is dominated by the rosette population since a given intensity must consist of many more small particles compared to few large particles. When vaccine was dried (without addition of an excipient), reconstituted, and then examined by DLS, intensity size distributions shifted towards larger diameters while number size distributions had much less regularity. The addition of trehalose with the vaccine before drying maintained both an intensity size distribution and number size distribution similar to liquid control vaccine.

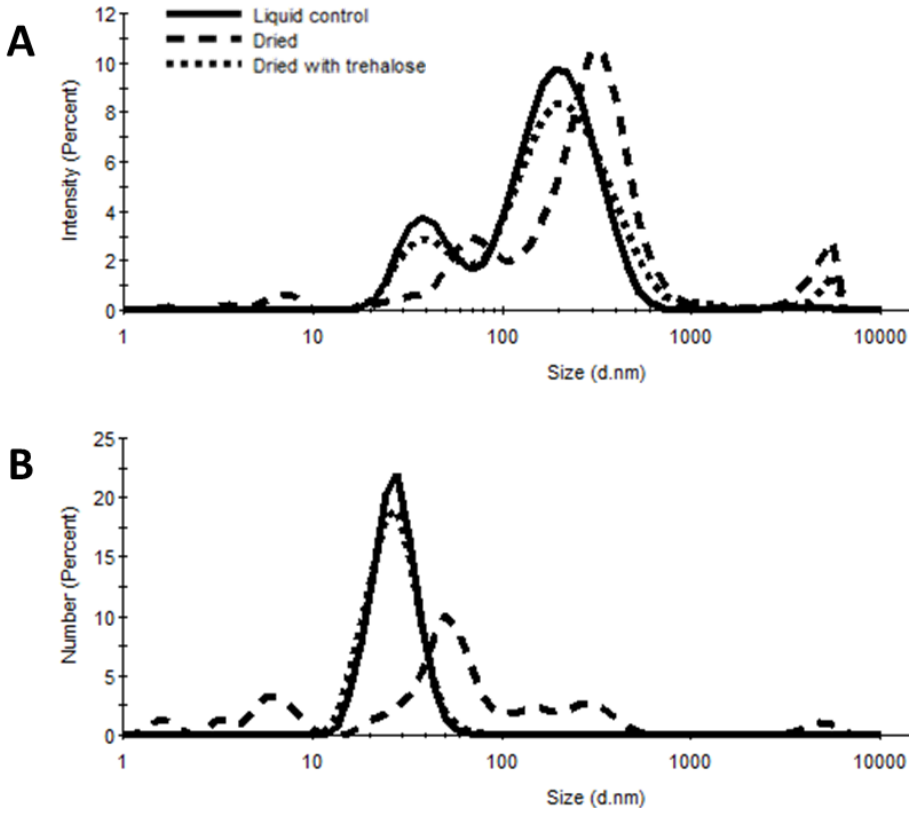

Figure 6.2. Dynamic light scattering of A/Texas/50/2012 vaccine. Representative graphs showing (A) intensity size distribution and (B) number size distribution both show a size increase of dried vaccine but the retention of the original distribution when trehalose as excipient is added to the vaccine. Each curve represents an average distribution (n=3), as computed by the manufacturer's software

6.3.3 Proteolysis assay

To probe whether HA undergoes a natural conformational change during drying, HA molecules were exposed to potential proteolysis by proteinase K.[18] Shown in Figure 6.3, unadulterated vaccine was not susceptible to enzyme digestion, as evident by the presence of the original full-length HA band (Lane C). Vaccine that had been heated, though, was not apparent on the gel after enzyme exposure, showing the ability of proteinase K to digest heat-treated vaccine (Lane D). Vaccine dried without an additional excipient was present on the protein gel (i.e., was not digested), leading to the interpretation that dried influenza vaccine does not undergo a conformational change. Several excipients were also included in the vaccine formulation before drying, showing no enzyme digestion when dried with trehalose or xylitol, yet xylose led to enzyme digestion after drying.

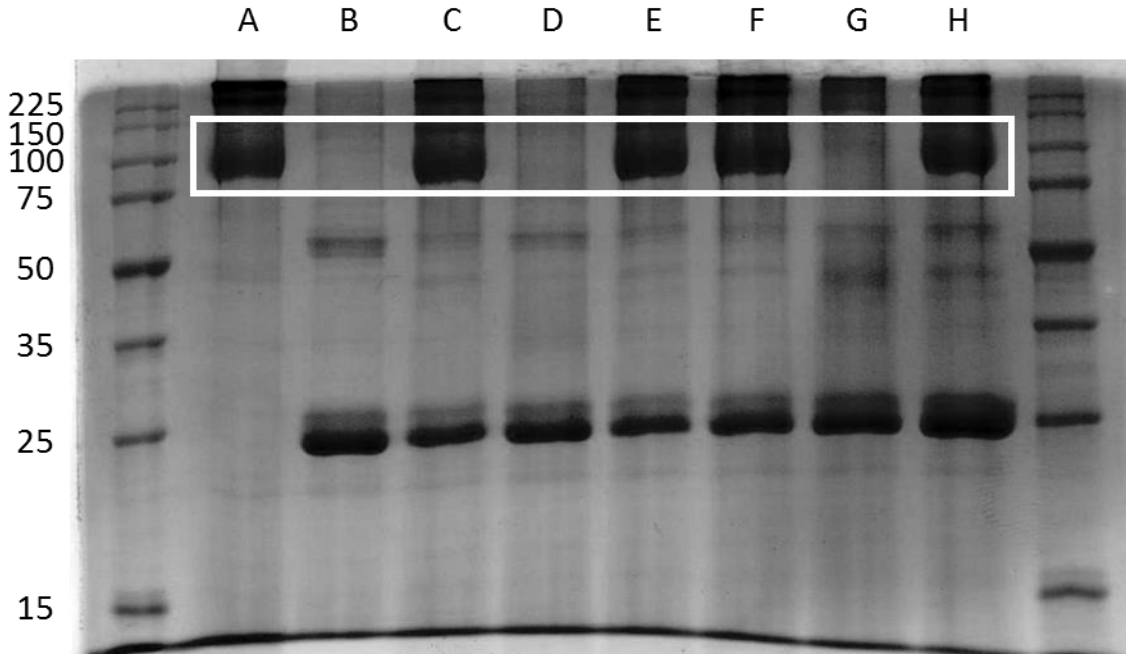

Figure 6.3. SDS-PAGE of A/Victoria/361/2011 vaccine after exposure to proteinase K enzyme digestion. The white box represents the location of undigested HA. The absence of a band within that area is indicative that HA underwent proteolysis due to a conformational change prior to proteinase K incubation. (A) Vaccine only and (B) enzyme only are shown for reference, while (C) native vaccine and (D) heated vaccine were incubated with proteinase K as negative and positive controls. Vaccine was also dried with (E) no added excipient, (F) trehalose, (G) xylose, and (H) xylitol. Numbers on the left side indicate the molecular weight in kDa of reference proteins run concurrently.

6.3.4 Circular dichroism

Evidence of secondary structure changes was probed with far-UV circular dichroism (CD) spectroscopy. Samples of three vaccine strains that had been dried, heated, or left as a liquid control were all examined with CD. CD data for no conditions were significantly different for A/Christchurch/16/2010(H1N1) and A/Texas/50/2012(H3N2) (Fig. 6.4a-b). Although the CD signal for A/Victoria/361/2011(H3N2) was nearly identical among the three conditions, the absorbance at 208 nm was significantly higher (Fig. 6.4c, Student's *t*-test, p= 0.03) for vaccine which had been dried without trehalose. This value indicates that there may be a slight perturbation to the protein secondary structure.

6.3.5 Intrinsic fluorescence

Further study of possible secondary structure changes was undertaken with the use of intrinsic protein fluorescence. λ_{max} values for refrigerated liquid control vaccine was compared to vaccine of the same strain that was dried with either trehalose or no added excipient or that had been heated in the liquid state. All experimental conditions yielded λ_{max} values that were statistically different than that of the liquid control (Fig 6.5, Student's *t*-test, $p < 0.05$), yet no differences were greater than 1.75 nm, which may represent a structure change slight enough to not have an effect on vaccine activity. All three strains showed an increase in λ_{max} values, indicating a slight opening of tertiary structure and generally more solvent exposure of aromatic amino acids. Vaccine dried without an added excipient showed decrease in λ_{max} values for A/Victoria/361/2011(H3N2) and A/Texas/50/2012(H3N2) but an increase in λ_{max} values for A/Christchurch/16/2010(H1N1). This discrepancy can be attributed to either varying locations of aromatic residues amongst the strains or varying behavior upon drying. Finally, all three vaccine strains, when dried with trehalose included in the formulation, showed a slight, less than 1 nm, decrease in λ_{max} values. These values were seen to be the same as liquid vaccine with trehalose added, thus attributing the decrease in λ_{max} values to a change in solvent nature. Fluorescence spectroscopy was not able to definitively rule out structural changes during drying, but there was no evidence of mass unfolding and denaturation.

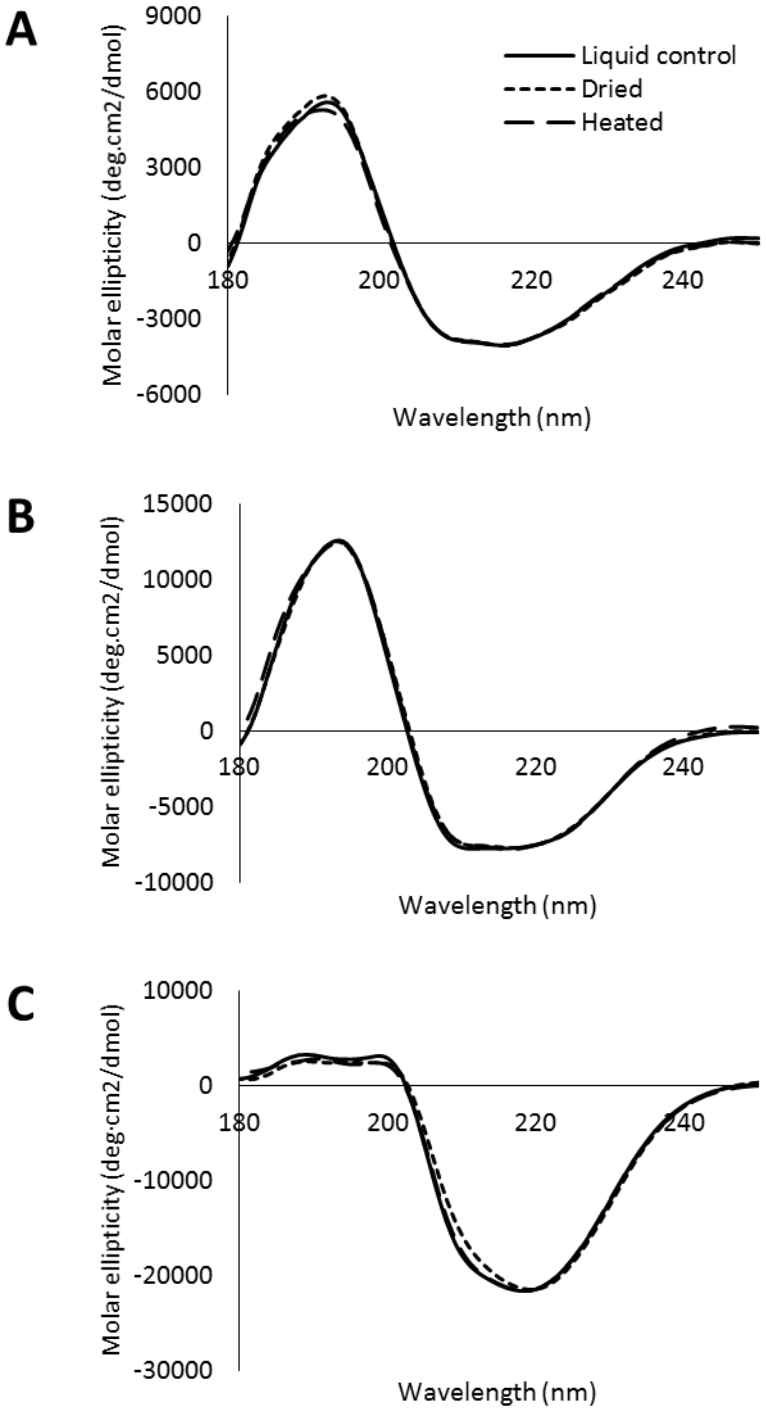

Figure 6.4. Far-UV circular dichroism spectra of (A) A/Christchurch/16/2010, (B) A/Texas/50/2012, and (C) A/Victoria/361/2011. Experimental conditions shown include liquid control (solid line), dried overnight at room temperature (short dashed line), and heated for three hours at 75°C (long dashed line). Spectra were acquired at 200 nm/min with 10 accumulations per sample. Each curve represents an arithmetic mean, n=3.

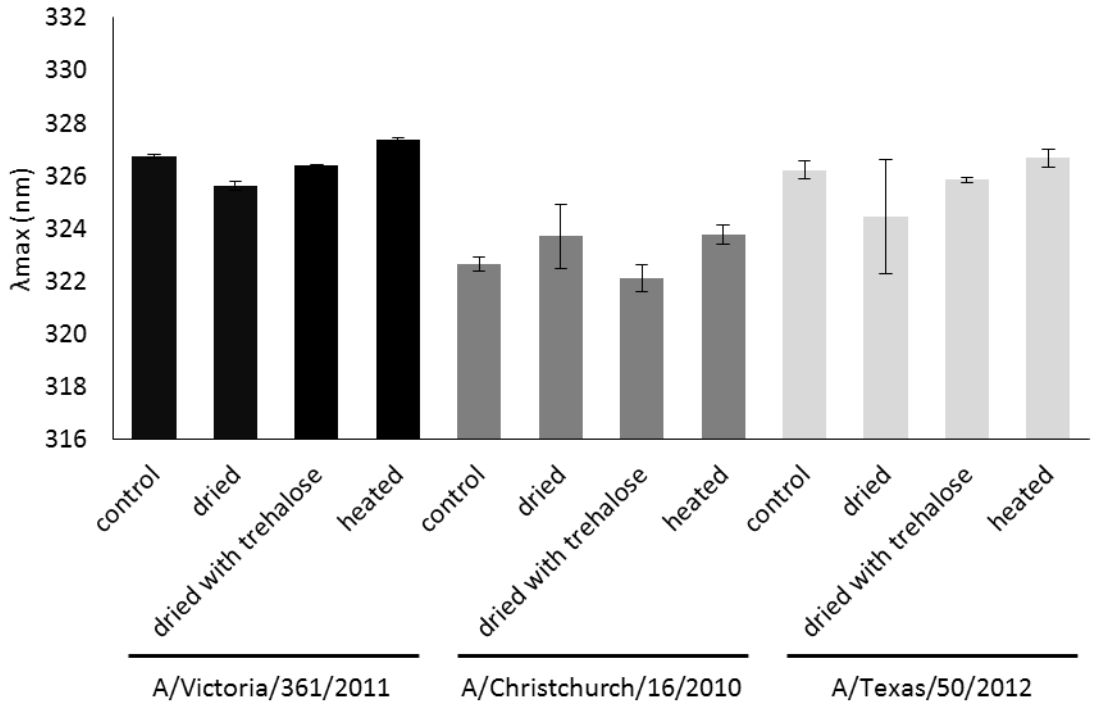

Figure 6.5. Wavelengths of peak intrinsic fluorescence for A/Victoria/361/2011 (n=3), A/Christchurch/16/2010 (n=9), and A/Texas/50/2012 (n=9) and at various experimental conditions. Data represent averages with standard deviation bars shown.

6.4 Discussion

Vaccine stability must be maintained for it to be efficacious once delivered regardless of its type or state. In the case of dissolving MN patches, that involves stability during drying. The work shown here was aimed at understanding more about how subunit influenza vaccine loses activity during air drying. Air drying is how dissolving MN patches for this work were produced and is therefore relevant but also may be distinct from other drying methods such as lyophilization or spray drying.

Commercial subunit influenza vaccine was utilized as it is a vaccine currently being developed for human clinical trials. This vaccine has been seen to lose roughly half of its activity after drying at room temperature for 24 hours, though without the presence of a protecting excipient. The two general hypotheses studied here was that there could be structural changes, either secondary or tertiary, or that there could be irreversible

aggregation occurring. A variety of techniques were utilized to monitor for these changes. When applicable, a well-known stabilizer, trehalose, was added to the vaccine formulation to determine its effect on vaccine activity retention and its interference with whichever degradation pathways were found to be influential.

When TEM was used to image vaccine samples, there were numerous rosettes of approximately 30-40 nm diameter, but also present were large spherical structures in the 100-300 nm diameter range seen in DLS scans. It appears that there were HA molecules protruding from their surfaces. We hypothesize that these structures are reformed after HA molecules are separated from intact virions and can be thought of as analogous to micelle formation in surfactant solutions. Although a limitation of standard TEM analysis is the necessity of drying samples on mesh grids, there was no observable difference in the size or frequency of large HA structures amongst vaccine samples that had remained liquid until processing for TEM or samples that had been dried with or without trehalose. Also noted visually were unstructured agglomerations of protein which did not appear correlated with drying condition. This was performed by visual analysis of a set of TEM images; further study with a higher resolution TEM instrument and image analysis may yield more conclusions. When vaccine samples were examined with DLS, there was clearly a population consisting of multimeric rosettes, which is typical of subunit influenza vaccine. These rosettes are formed because of the interaction of multiple HA molecules' hydrophobic transmembrane regions.[219] Alongside the rosette population, there was also a larger population (i.e., 30 – 40 nm) that was rather large to be intact virions (i.e., ~80 – 100 nm[220]) that were not broken apart during vaccine production.

Although there was no visual difference between vaccine conditions examined with TEM, there was a marked difference between liquid vaccine and dried vaccine measured by DLS. While liquid control vaccine displayed two consistent, distinct peaks, vaccine which had been dried without excipients and reconstituted gave a signal which was not as smooth and was relatively variable for multiple samples. It is unclear what form the new species were, but the lack of or shift of the rosette peak was apparent in drying conditions associated with a loss of vaccine activity seen previously. Vaccine dried with trehalose, on the other hand, displayed the same two DLS peaks as liquid vaccine. This can be attributed to trehalose's ability to form a glass phase during drying that is extremely viscous and

immobilizes entities contained inside it.[89] Saccharides are also known as kosmotropic agents.[221-223] Kosmotropic agents, in general, strongly interact with water and are excluded from the solvation layer of proteins.[224] This leads to a decrease in water diffusion around the surface of the protein, lowering the flexibility and stabilizing the protein.[225] The kosmotropic nature of trehalose likely decreases protein-protein interaction causing an impediment to aggregation. Further analysis of vaccine particle sizes distributions and species could be carried out with a gel filtration experiments. If these species could be separated, then the activity of various species could be tested.

This kosmotropic behavior was also evident when vaccine was probed for intrinsic fluorescence. Overall, there was little, but statistically significant, difference in peak intensity wavelength, λ_{max}, amongst the experimental conditions tested. Vaccine dried with trehalose showed a blue-shift of λ_{max} indicating a less exposed environment of the HA protein's aromatic residues.[156] Control samples of liquid vaccine with added trehalose also showed similar drops in λ_{max}, suggesting that the change seen in vaccine dried with trehalose is caused by the presence of trehalose in solution after rehydration. As for vaccine dried without an excipient, results were more varied. λ_{max} increased for one strain and decreased for the other two. Notably, the two strains that behaved similarly were A/Victoria/361/2011 and A/Texas/50/2012, which have a 98.6% sequence identity, so the corresponding results are not surprising. The differing fluorescence behavior could be attributed to different numbers of tyrosine and tryptophan between the H1N1 and H3N2 strains. Finally, heated samples showed a consistent red-shift of λ_{max} consistent with a general loosening of tertiary structure and exposure of aromatic residues.[156]

Additional inspection of secondary structure was undertaken with CD spectroscopy. Similarly to intrinsic fluorescence spectroscopy, there were little to no differences found between experimental groups. It is well known that HA undergoes a conformational change at low endosomal pH or elevated temperatures.[226] It was expected that heated vaccine would show a change in CD spectrum compared to control vaccine, yet this was not seen. Vaccine availability limited this work to far-UV CD, but given a large enough vaccine stock for concentrated samples, near-UV CD may be able to detect more subtle changes to protein structure. When vaccine was dried, there were no spectrum changes for A/Texas/50/2012 and A/Christchurch/16/2010(H1N1), but there was

a slight change in A/Victoria/361/2011 when dried, indicative of a loss of α-helical nature.[227] It is unclear with the techniques utilized to pinpoint the exact location of this structure change, whether it is in a region containing antibody-generating epitopes

Besides the two spectroscopic methods already discussed, the fusion-based conformational change of HA can be monitored by enzyme proteolysis.[16] Native-conformation HA is resistant while HA in the fusogenic state is susceptible to cleavage of the HA1 polypeptide near its disulfide connection to HA2.[18] As expected, non-heated and heated vaccine samples were resistant and susceptible to cleavage, respectively. The test condition of dry vaccine was shown to be resistant to proteolytic cleavage. The stresses of drying does not affect HA in a way that would cause a conformational change detected by this assay. Therefore, the stabilizing effects of trehalose were not needed, and trehalose addition did not have an effect on HA cleavage. Besides trehalose, we chose to test vaccine drying with xylitol and xylose as well, since they performed very well and very poorly, respectively, during early formulation screening experiments.[195] The reason for vaccine activity loss during drying in the presence of xylose is apparently associated with the induction of a conformational change. Xylose is a reducing sugar, which implicates a likely reaction with the side chain of an amino acid residue. Influenza vaccine cannot be stabilized by the inclusion of any saccharide, or even any non-reducing saccharide. For example, lactose, a reducing sugar, was a successful stabilizer, suggesting that there are other factors involved in the ability of a sugar to stabilize HA during drying.

With this insight into the mechanisms of influenza vaccine activity loss, we hope that formulation knowledge can be applied for various vaccines. Excipient properties appearing to contribute to the ability of an excipient to stabilize influenza vaccine are a kosmotropic nature and the ability to form a glass phase to prevent aggregation. As these do not depend directly on the specific protein, other subunit vaccines could possibly work well in similar formulations. A limitation of this work includes the lack of information regarding whether there were any specific interactions between trehalose and the protein during drying. Specific interactions could make the application of top stabilizing formulations less generalizable to multiple vaccines or even strains of the same vaccine, as the amino acid sequence of influenza vaccines can change from year to year. Another limitation is the unknown nature of the aggregation pathways during drying of vaccine

without a stabilizer. These aggregates could change over time without an excipient or could also be affected by possible changes to the excipient environment over time during storage.[172] The protein secondary and tertiary structure remained unchanged in most cases during drying. This consistent structure would benefit the production of antibodies to the correct epitopes and ensure the vaccine would be protective against a possible infection.

6.5 Conclusion

Vaccine stability is vitally important to immunization campaigns due to both the large percentage of overall cost the cold-chain represents as well as vaccine wastage and non-efficacious doses due to faults in the cold-chain during storage and transportation. Microneedle patches represent a promising vaccine delivery method with numerous advantages over current vaccination methods, but the drying of vaccines during production and storage poses a barrier to commercialization. This work, which focused on subunit influenza vaccine, aimed to determine the mechanism(s) of vaccine activity loss during air drying. The predominant change to vaccine structure was aggregation as opposed to significant secondary or tertiary structure changes to the influenza hemagglutinin protein. Formulations that prevent hemagglutinin aggregation are expected to stabilize influenza vaccine doses in a dried state and possibly allow for their removal from the cold-chain. Our hope is that this work will further the development of microneedle patches for influenza vaccine as well as other vaccines which could benefit from the advantages of microneedle patch technology.

6.6 Acknowledgments

We thank Novartis Vaccines and Diagnostics for graciously provided monovalent influenza vaccine stock. This work was supported in part by the National Institutes of Health. We thank Miraj Desai for his work on this project. The work was carried out in the Center for Drug Design, Development and Delivery, and the Institute for Bioengineering and Bioscience at the Georgia Institute of Technology. We thank Dr. Hong Yi at Emory University for microscopy work. This research project was supported in part by the Robert

P. Apkarian Integrated Electron Microscopy Core of Emory University with the National Institutes of Health grant S10RR025679. Matthew Mistilis, Andreas Bommarius, and Mark Prausnitz are inventors of patent(s) that have been or may be licensed to companies developing microneedle-based products, and Mark Prausnitz is a paid advisor to companies developing microneedle-based products and is a founder/shareholder of companies developing microneedle-based products. This potential conflict of interest has been disclosed and is overseen by Georgia Tech and Emory University.

CHAPTER VII

DISCUSSION

A novel drug delivery system under development is the dissolving microneedle patch. When compared to the standard hypodermic needle and syringe currently used for most immunizations, MN patches provide numerous benefits. From the patient's perspective, MN patches are a painless alternative to a hypodermic injection. There is also the possibility that patients will be able to self-administer vaccinations without the assistance or observation of a healthcare worker. The ability to pick up a vaccine MN patch at a pharmacy or grocery store for oneself and one's family to be used painlessly at home is likely to raise a population's acceptance to receiving a vaccine and increase vaccination coverage. While there will be biohazardous waste, it is not sharps waste, thus simplifying waste disposal. Also, there is evidence that immunization by MN patch is more immunogenic than an intramuscular injection.[69] This opens two possibilities. Either delivery of the same dose as an intramuscular injection generates an improved immune response, or the dose of vaccine delivery can be lowered, thus saving cost and spreading the current vaccine production over more doses.

A key to simplifying vaccination logistics is the capability of MN patches to be stored and transported outside of a controlled cold-chain. Most commercial vaccines must remain refrigerated or frozen until immediately before delivery. This can be cumbersome and very expensive, especially in developing countries, where the cold-chain is much less reliable.[228] Even in developed countries, cold-chain storage adds a significant cost to any vaccine program. Vaccine outside of the cold-chain by faulty or unavailable equipment as well as by human error is typically discarded. This vaccine waste is an expensive, but avoidable cost for vaccine programs.

MN patches are able to maintain vaccine activity outside of the cold-chain by means of keeping the vaccine in a dried state, where degradation pathways can be significantly slowed or eliminated. The primary method of modulating vaccine stability is through MN patch formulation development. Excipients added to the solutions used in the patch production process can have a positive or negative effect on vaccine stability. They also

must be safe for use in humans and retain the patch's ability to insert into skin and dissolve within a reasonable time.

Working towards a thermostable influenza dissolving MN patch, this work began with a screen of factors possibly affecting influenza vaccine stability during drying, followed by the implementation of several leading candidate formulations in the production of MN patches which were stressed by various means, stored at room temperature for extended periods, and used to vaccinate mice as a verification of *in vivo* immunogenicity.

Formulation screening was performed on flat polydimethylsiloxane (PDMS) and stainless steel (SS) surfaces referred to as "chips". These chips represented the surface vaccine is dried on when making dissolving and coated MN, respectively. The use of chips greatly increased the throughput of screening compared to full MN patches, as well as allowed for isolation of particular variables, since MN patch production is a more intricate process. The rate of initial drying, from ten minutes to one hour, as dictated by drying temperature, did not have a significant impact in most cases so room temperature drying was chosen to avoid complicating patch production. Concurrently, buffer salts were tested. Phosphate buffered saline, which is what commercial influenza vaccine is delivered in, did not perform well, likely owing to a drop in pH during drying. This is commonly seeing during freezing of protein solutions,[83]thus this buffer should be avoided. With both (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) and ammonium acetate being successful at maintaining vaccine activity, a choice of buffer needed to be made. Ammonium acetate was chosen as the buffer for all future stability studies in part because of its stabilizing capability but also because it is a volatile salt, where its constituent ions, acetate and ammonium, form two volatile compounds, acetic acid and ammonia, respectively. The hypothesis is that the presence of salts in the final matrix may lead to unfavorable interactions, thus ammonium acetate was chosen to prevent a possibly damaging scenario.

A relatively large screen of excipient compounds contained a range of good stabilizers and bad stabilizers. Unsurprisingly, carbohydrates dominated the top list of good stabilizers. It's well known that carbohydrates have stabilizing properties for proteins in a dry state.[191] Many of them form an amorphous glassy phase when dried that has such high viscosity that anything in the solution initially is subsequently confined and

immobilized. Degradation pathways that involve protein mobility are therefore impeded. Many carbohydrates also are capable of interacting favorably with proteins via their numerous carboxyl groups.[192]

Previous work in our lab has shown that certain stabilizing compounds, such as trehalose and sucrose, tend to crystallize over time, which is unfavorable for protein stability.[172] Also in our lab and in the literature[189], combinations of stabilizers have performed better over time than either of the constituent stabilizers did by themselves. Here stabilizer combinations were screened, and it was found that even stabilizers that performed well during initial vaccine drying lost activity over time, whereas certain combinations of those stabilizers fully maintained vaccine activity. These top stabilizer combinations were trehalose/sucrose, sucrose/arginine, and arginine/heptagluconate. The likely cause of the combinations' success is the inhibition of crystallization by the presence of "impurities", the other excipients.[229]

Once the large number of possible formulations had been narrowed down, the top formulations were utilized to produce monovalent and trivalent influenza vaccine dissolving MN patches. The formulations were largely successful in this regard as well. High stability over a period of eighteen months at room temperature was observed, which is very promising for feasibility of storage of commercial MN patches outside of the cold-chain. Since influenza is typically manufactured as a seasonal, annual vaccine, eighteen months of stability exceeds the necessary shelf-life. In the case of a pandemic vaccine to be stockpiled, though, a longer shelf-life may be necessary. The patches with arginine/heptagluconate as stabilizers generally performed the best, but all patches retained most of their activity. Even patches produced with a vaccine solution lacking a stabilizer retained vaccine activity during storage after an initial loss during production. This leads to the hypothesis that the primary mode of action of stabilizing excipients is to protect the vaccine payload from the stresses of drying during production. After the patches are complete, the sucrose and polyvinyl alcohol making up the bulk of the MNs and backing are sufficient to preserve vaccine activity during storage.

The robustness of the influenza vaccine MN patches developed here has several ramifications. The resistance to vaccine and patch degradation during irradiation by electron beam opens up possibilities for terminal sterilization. This would allow for a

simpler and cheaper commercial production process compared to an aseptic production. Also, the patches' ability to withstand both freeze-thaw stresses and elevated temperatures for extended periods lessens the concern that MN patches may deliver a suboptimal dose of vaccine after extensive storage and transportation.

There was already evidence that vaccination via MN patch could be more immunogenic compared to an identical dose delivery via intramuscular or intradermal. In our immunogenicity study with fresh and aged patches, patches elicited higher antibody titers than an equivalent dose delivered intradermally. Again, this would permit for either a stronger immune response with a standard influenza vaccine dose or the ability to deliver less vaccine to generate a sufficient response, allowing the current manufacturing quantity to be used to vaccinate more people.

Finally, we took a closer look at the mechanism of influenza vaccine activity loss. Further knowledge of the mechanism could aid in the development of MN patches for other vaccines, specifically those of the subunit variety. It was found that the primary mode of activity loss was the aggregation of viral proteins, preventing antibody recognition of those proteins and likely also preventing an immune response in the form of antibody generation. Investigation by circular dichroism, intrinsic fluorescence, and enzyme digestion showed little if any change to the immunogenic protein, hemagglutinin, during drying, even without a stabilizer included in the formulation. There were, however, changes in the hydrodynamic size distribution, as seen with dynamic light scattering. Addition of trehalose in dried samples led to a size distribution comparable to liquid samples, typically at full or near-full activity. Here, the inclusion of stabilizers mitigated much of the aggregation during drying.

CHAPTER VIII

RECOMMENDATIONS

8.1 Extension of successful formulations to other vaccines

This work was performed solely with influenza subunit vaccine. While stability of this vaccine in microneedle patches outside of the cold-chain was achieved, there are a myriad of vaccine types for different diseases. The ability to incorporate more vaccines into microneedle patches will benefit the fight against vaccine preventable illnesses and deaths as well as to help further the reach of this delivery method. Influenza subunit vaccine is composed primarily of hemagglutinin and neuraminidase proteins. While these are membrane proteins, the stabilizing properties of the stabilizing formulations developed in this work should translate well to the stabilization of other proteins, whether part of a vaccine or other therapeutic. The glassy phase trehalose likely forms during air drying is thought to prevent irreversible aggregation of proteins upon drying is relatively independent of the drug payload assuming the loading drug is of relatively low weight percent of overall solids content.

The ability of the formulations developed in this work to protect other types of vaccines is less certain. For example, whole inactivated vaccines, such as influenza and a variety of polio vaccine, are intact virions, whose size retention is sensitive to external effects such as osmotic pressure.[230] It is possible that trehalose would help protect the proteins involved but may not be capable of maintaining all conditions needed for full vaccine activity. The antigens for influenza and polio vaccines are a membrane bound protein and an exterior protein capsid[220], respectively, so the capacity for certain formulations to stabilize different structures must be verified. In addition to protein structural changes, osmotic pressure and spatial confinement can greatly damage larger viral structures.[122] A step further in stabilization difficulty would be live attenuated vaccines such as measles, where the viral particles must retain the ability to replicate within the recipient to generate an appropriate immune response. Therefore not only must the

overall structure persist intact, but the interior machinery needs to be preserved enough for replication to proceed after delivery. Vaccines consisting of nucleic acids such as DNA plasmids bring a whole new set of parameters to stability. DNA supercoiling is strongly correlated to activity and stability.[231] These vaccine would require separate study.

8.2 Inclusion of adjuvants in microneedle formulations

Adjuvants are substances which are modulates or increases the immune response to a given antigen. Vaccine delivery into the skin can give an increased immune response compared to intramuscular injection, and adjuvant will likely only increase a vaccine's potency. The two most popular vaccine adjuvants currently in human use are aluminum salts and squalene. The exact mechanisms of adjuvant action are still under investigation, but their inclusion in vaccine dosages can allow for dose sparing or more effective vaccines. Thus they are of great interest for inclusion into microneedle patch designs. Any excipient included in a microneedle patch formulation could possibly effect the outcome of the drug delivery. Aluminum salts, if included at a significant weight percent could affect the integrity of the microneedles or the ability of the other excipients to stabilize the vaccine to be delivered. The microneedles can also become rubber or brittle, preventing insertion into the skin.

Squalene is not an adjuvant in its own right, but is combined with surfactants to produce and oil-in-water emulsification. It is unlikely that including an emulsification in the vaccine formulation will allow for the creation of microneedles, but it is worth investigating whether the components, such as squalene, Tween 80, and Span 85 found in the adjuvant MF59 by Novartis, can be incorporated into microneedles and delivered concurrently with a vaccine.

There are other compounds being studied as potential adjuvants for human vaccines. These include cytokines, carbohydrates, and bacterial peptidoglycans. If these progress through further development, they would be promising adjuvants to include in the production of microneedle patches for vaccine delivery. Cytokines are small proteins which would likely be stabilized similar to viral antigens proteins. Carbohydrates are already commonplace in dry biopharmaceutical preparations, and peptidoglycans are

polymers of sugars and amino acids. Both carbohydrates and peptidoglycans would likely assimilate well into microneedle formulations.

8.3 Rapid dissolution of microneedles

There have been several studies investigating the feasibility of vaccine delivery via dissolving microneedle patches. A noted concern from patch recipient is the long wear time needed to allow for microneedle dissolution and delivery of the vaccine.[232] Decreasing the time required for complete delivery will both ease the administration procedure and increase the recipient's acceptability.

A possible method of decreasing the wear time is to include a superdisintegrant into the formulation.[233] One mechanism of action is to promote the penetration of moisture into the microneedle through wicking, breaking it into small pieces and creating more surface area to allow more moisture penetration. Another mechanism is to swell when in contact with water, overcoming the adhesiveness of other compounds present. Common superdisintegrants are crosslinked versions of cellulose, starch, or polyvinylpyrrolidone. They would be added to a small degree in either the initial vaccine fill and/or the polymer backing solution.

Another method of decreasing the wear time would be to add another fill step to the production process. This step would add a layer between the layer at the tip of the microneedle containing the vaccine payload and the polymer backing. The added layer would contain compounds such as superdisintegrants or salts that dissolve nearly immediately upon insertion into the skin. The dissolution of this layer will separate microneedle from the back, leaving the microneedle to dissolve at an arbitrary rate inside the skin after the backing is removed very soon after microneedle patch application.

CHAPTER IX

CONCLUSION

The development of vaccines within the last century has helped bring an era of extended lifespans and less suffering from what are now known as vaccine-preventable diseases. There will undoubtedly be more diseases to earn that label, but there is still work to be done with currently proven and approved vaccines. Microneedle patches are a very promising novel drug delivery system that can be utilized to improve vaccinations. In addition to the benefits including absence of biohazardous sharps waste, painless self-administration, and increased immunogenicity, MN patches could allow for the storage and transportation of vaccine doses outside of the cold-chain. Through systematic screening of MN patch formulations, we have developed a MN patch that maintained influenza vaccine activity when stored for up to a year and a half at room temperature. In addition, these patches were shown to be robust in ways that would benefit production and their resistance to storage errors. Through in-depth analysis of vaccine inactivation during drying, the formulations developed here may likely assist in the advancement of MN patch technology for the delivery of other vaccines besides influenza vaccine. The impact of the work described here can best be characterized by its applicability to a range of systems. Lyophilized protein stability is still a relatively empirical field where novel formulations are welcome. The development of microneedle systems is much newer than lyophilization, and the stability and formulation considerations are much less well-known for this novel drug delivery method. Formulations which both maintain vaccine activity and form proper microneedle patches are still under development. This work describes such formulations and gives more support to the eventual commercialization and implementation of microneedle to solve healthcare problems. Much work is remains to be done to rid ourselves of vaccine-preventable diseases, but MN patches could help reach this goal.

REFERENCES

1. Cox, N.J. and K. Subbarao, *Influenza.* The Lancet, 1999. **354**(9186): p. 1277-1282.

2. Ghedin, E., et al., *Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution.* Nature, 2005. **437**(7062): p. 1162-1166.

3. Couch, R.B. and J.A. Kasel, *Immunity to Influenza in Man.* Annual Review of Microbiology, 1983. **37**(1): p. 529-549.

4. Skehel, J.J. and D.C. Wiley, *RECEPTOR BINDING AND MEMBRANE FUSION IN VIRUS ENTRY: The Influenza Hemagglutinin.* Annual Review of Biochemistry, 2000. **69**(1): p. 531-569.

5. Palese, P. and M.L. Shaw, *Orthomyxoviridae: The Viruses and Their Replication*, in *Fields Virology*, D.M. Knipe and P.M. Howley, Editors. 2007, Wolters Lippincott: Philadelphia.

6. Matsuzaki, Y., et al., *Clinical Features of Influenza C Virus Infection in Children.* Journal of Infectious Diseases, 2006. **193**(9): p. 1229-1235.

7. Horwood, F. and M. J., *Pneumococcal and influenza vaccination: current situation and future prospects.* Thorax, 2002. **57**(Supplement 2): p. II24-II30.

8. Coudeville, L., et al., *Relationship between haemagglutination-inhibiting antibody titres and clinical protection against influenza: development and application of a bayesian random-effects model.* Bmc Medical Research Methodology, 2010. **10**.

9. Kitler, M.E., P. Gavinio, and D. Lavanchy, *Influenza and the work of the World Health Organization.* Vaccine, 2002. **20, Supplement 2**: p. S5-S14.

10. Quinn, E., M. Jit, and A.T. Newall, *Key issues and challenges in estimating the impact and cost-effectiveness of quadrivalent influenza vaccination.* Expert Review of Pharmacoeconomics & Outcomes Research, 2014. **14**(3): p. 425-435.

11. Nobusawa, E., et al., *Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses.* Virology, 1991. **182**(2): p. 475-485.

12. Liu, N., et al., *Mutations in influenza virus replication and transcription: detection of amino acid substitutions in hemagglutinin of an avian influenza virus (H1N1).* The FASEB Journal, 2009. **23**(10): p. 3377-3382.

13. Higgins, C.D., et al., *Influence of a heptad repeat stutter on the pH-dependent conformational behavior of the central coiled-coil from influenza hemagglutinin HA2.* Proteins: Structure, Function, and Bioinformatics, 2014. **82**(9): p. 2220-2228.

14. Weis, W., et al., *Structure of the influenza-virus hemaglutinin complexed with its recepter, sialic acid.* Nature, 1988. **333**(6172): p. 426-431.

15. Bullough, P.A., et al., *Structure of influenza haemagglutinin at the pH of membrane fusion.* Nature, 1994. **371**(6492): p. 37-43.

16. Carr, C.M. and P.S. Kim, *A spring-loaded mechanism for the conformational change of influenza hemagglutinin.* Cell, 1993. **73**(4): p. 823-832.

17. Doms, R.W., A. Helenius, and J. White, *Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change.* Journal of Biological Chemistry, 1985. **260**(5): p. 2973-2981.

18. Carr, C.M., C. Chaudhry, and P.S. Kim, *Influenza hemagglutinin is spring-loaded by a metastable native conformation.* Proceedings of the National Academy of Sciences, 1997. **94**(26): p. 14306-14313.

19. Dreyfus, C., et al., *Highly Conserved Protective Epitopes on Influenza B Viruses.* Science, 2012. **337**(6100): p. 1343-1348.

20. Amorij, J.P., et al., *Development of stable influenza vaccine powder formulations: Challenges and possibilities.* Pharmaceutical Research, 2008. **25**(6): p. 1256-1273.

21. Lee, M.-k., et al., *Analysis of genome integrity of influenza virus in formaldehyde-inactivated split vaccines.* Genes & Genomics, 2014. **36**(5): p. 633-641.

22. Budowsky, E.I., Y.A. Smirnov, and S.F. Shenderovich, *Principles of selective inactivation of viral genome. VIII. The influence of β-propiolactone on immunogenic and protective activities of influenza virus.* Vaccine, 1993. **11**(3): p. 343-348.

23. Beyer, W.E.P., et al., *Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy: A meta-analysis.* Vaccine, 2002. **20**(9–10): p. 1340-1353.

24. Abrahamson, J.S., *Intranasal, cold-adapted, live, attenuated influenza vaccine.* Journal of Pediatric Infectious Diseases, 1999. **18**: p. 1103-1104.

25. Huckriede, A., et al., *The virosome concept for influenza vaccines.* Vaccine, 2005. **23, Supplement 1**(0): p. S26-S38.

26. Zuccotti, G.V. and V. Fabiano, *Influvac, a trivalent inactivated subunit influenza vaccine.* Expert Opinion on Biological Therapy, 2011. **11**(1): p. 89-98.

27. Wharton, S.A., et al., *Electron microscopy of antibody complexes of influenza virus haemagglutinin in the fusion pH conformation.* EMBO J, 1995. **14**(2): p. 240-6.

28. Hunter, R.L., *Overview of vaccine adjuvants: present and future.* Vaccine, 2002. **20, Supplement 3**(0): p. S7-S12.

29. Cox, J.C. and A.R. Coulter, *Adjuvants—a classification and review of their modes of action.* Vaccine, 1997. **15**(3): p. 248-256.

30. Annersten, M. and A. Willman, *Performing subcutaneous injections: A literature review.* Worldviews on Evidence-Based Nursing, 2005. **2**(3): p. 122-130.

31. Combadiere, B. and C. Liard, *Review of transcutaneous and intradermal vaccination.* Human Vaccines, 2011. **7**(8): p. 811-827.

32. Kupper, T.S. and R.C. Fuhlbrigge, *Immune surveillance in the skin: Mechanisms and clinical consequences.* Nature Reviews Immunology, 2004. **4**(3): p. 211-222.

33. Adib-Hajbaghery, M. and M.S. Lotfi, *Behavior of healthcare workers after injuries from sharp instruments.* Trauma monthly, 2013. **18**(2): p. 75-80.

34. Simonsen, L., et al., *Unsafe injections in the developing world and transmission of bloodborne pathogens: A review.* Bulletin of The World Health Organization, 1999. **77**(10): p. 789-800.

35. Kim, Y.C., et al., *Delivery systems for intradermal vaccination.* Current topics in microbiology and immunology, 2012. **351**: p. 77-112.

36. Blenkharn, J.I. and C. Odd, *Sharps Injuries in Healthcare Waste Handlers.* Annals of Occupational Hygiene, 2008. **52**(4): p. 281-286.

37. Giudice, E.L. and J.D. Campbell, *Needle-free vaccine delivery.* Advanced Drug Delivery Reviews, 2006. **58**(1): p. 68-89.

38. MedImmune Vaccines Inc. *About FluMist*. 2011 1/12/12]; Available from: http://hcp.flumist.com/influenza-vaccine-about.

39. Esposito, S., et al., *Immunogenicity and safety of intradermal influenza vaccine in children.* Vaccine, 2011. **29**(44): p. 7606-7610.

40. Kenney, R.T., et al., *Dose sparing with intradermal injection of influenza vaccine.* New England Journal of Medicine, 2004. **351**(22): p. 2295-2301.

41. Skountzou, I., et al., *Transcutaneous immunization with inactivated influenza virus induces protective immune responses.* Vaccine, 2006. **24**(35-36): p. 6110-6119.

42. Van Kampen, K.R., et al., *Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans.* Vaccine, 2005. **23**(8): p. 1029-1036.

43. World Health Organization. *Intradermal Influenza (Flu) Vaccination*. 2011 [cited 03/26/12; Available from: http://www.cdc.gov/flu/protect/vaccine/qa_intradermal-vaccine.htm.

44. Fluzone, *Fluzone Intradermal (Influenza Virus Vaccine): Facts at a glance*, Fluzone, Editor. 2011.

45. Gorse, G.J., et al., *Intradermally-administered influenza virus vaccine is safe and immunogenic in healthy adults 18–64 years of age.* Vaccine, 2013. **31**(19): p. 2358-2365.

46. PharmaJet® Inc, *FDA Approves Use of AFLURIA® Influenza Vaccine with PharmaJet's Needle-Free Injector*. 2014, PharmaJet® Inc: Golden, CO, USA.

47. McAllister, L., et al., *Needle-free jet injection for administration of influenza vaccine: a randomised non-inferiority trial.* The Lancet. **384**(9944): p. 674-681.

48. Belshe, R.B., et al., *Serum antibody responses after intradermal vaccination against influenza.* The New England Journal Of Medicine, 2004. **351**(22): p. 2286-2294.

49. Gill, H.S., et al., *Effect of microneedle design on pain in human volunteers.* Clinical Journal of Pain, 2008. **24**(7): p. 585-594.

50. Glenn, G.M., et al., *Transcutaneous immunization: A human vaccine delivery strategy using a patch.* Nature Medicine, 2000. **6**(12): p. 1403.

51. Stingl, G., *DENDRITIC CELLS OF THE SKIN.* Dermatologic Clinics, 1990. **8**(4): p. 673-679.

52. Kim, Y.C., et al., *Improved influenza vaccination in the skin using vaccine coated microneedles.* Vaccine, 2009. **27**(49): p. 6932-6938.

53. Norman, J., et al., *Reliability and accuracy of intradermal injection by Mantoux technique, hypodermic needle adapter, and hollow microneedle in pigs.* Drug Delivery and Translational Research, 2014. **4**(2): p. 126-130.

54. Nordquist, L., et al., *Novel microneedle patches for active insulin delivery are efficient in maintaining glycaemic control: An initial comparison with subcutaneous administration.* Pharmaceutical Research, 2007. **24**(7): p. 1381-1388.

55. Wermeling, D.P., et al., *Microneedles permit transdermal delivery of a skin-impermeant medication to humans.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(6): p. 2058-2063.

56. Badran, M.M., J. Kuntsche, and A. Fahr, *Skin penetration enhancement by a microneedle device (Dermaroller (R)) in vitro: Dependency on needle size and applied formulation.* European Journal of Pharmaceutical Sciences, 2009. **36**(4-5): p. 511-523.

57. Qian, S.S., et al., *Enhanced Penetration of Sinomenine Fomulations Following Skin Pretreatment with a Polymer Microneedle Patch.* Latin American Journal of Pharmacy, 2014. **33**(3): p. 464-469.

58. Yan, G.A., et al., *Evaluation needle length and density of microneedle arrays in the pretreatment of skin for transdermal drug delivery.* International Journal of Pharmaceutics, 2010. **391**(1-2): p. 7-12.

59. Kim, Y.-C., J.-H. Park, and M.R. Prausnitz, *Microneedles for drug and vaccine delivery.* Advanced Drug Delivery Reviews, 2012. **64**(14): p. 1547-1568.

60. Gill, H.S. and M.R. Prausnitz, *Coated microneedles for transdermal delivery.* Journal of Controlled Release, 2007. **117**(2): p. 227-237.

61. Cormier, M., et al., *Transdermal delivery of desmopressin using a coated microneedle array patch system.* Journal of Controlled Release, 2004. **97**(3): p. 503-511.

62. Chabri, F., et al., *Microfabricated silicon microneedles for nonviral cutaneous gene delivery.* Br J Dermatol, 2004. **150**(5): p. 869-77.

63. Chen, X.F., et al., *Dry-coated microprojection array patches for targeted delivery of immunotherapeutics to the skin.* Journal of Controlled Release, 2009. **139**(3): p. 212-220.

64. Kim, Y.C., et al., *Formulation of microneedles coated with influenza virus-like particle vaccine.* Aaps Pharmscitech, 2010. **11**(3): p. 1193-1201.

65. McGrath, M.G., et al., *Determination of parameters for successful spray coating of silicon microneedle arrays.* International Journal of Pharmaceutics, 2011. **415**(1-2): p. 140-149.

66. Gill, H.S. and M.R. Prausnitz, *Coating formulations for microneedles.* Pharmaceutical Research, 2007. **24**(7): p. 1369-1380.

67. Widera, G., et al., *Effect of delivery parameters on immunization to ovalbumin following intracutaneous administration by a coated microneedle array patch system.* Vaccine, 2006. **24**(10): p. 1653-1664.

68. Sullivan, S.P., et al., *Dissolving polymer microneedle patches for influenza vaccination.* Nature Medicine, 2010. **16**(8): p. 915-U116.

69. Vassilieva, E.V., et al., *Improved immunogenicity of individual influenza vaccine components delivered with a novel dissolving microneedle patch stable at room temperature.* Drug Deliv Transl Res, 2015. **5**(4): p. 360-71.

70. van der Maaden, K., W. Jiskoot, and J. Bouwstra, *Microneedle technologies for (trans)dermal drug and vaccine delivery.* Journal of Controlled Release, 2012. **161**(2): p. 645-655.

71. Mattos, C. and D. Ringe, *Proteins in organic solvents.* Current Opinion in Structural Biology, 2001. **11**(6): p. 761-764.

72. Maa, Y.F., et al., *Stabilization of alum-adjuvanted vaccine dry powder formulations: Mechanism and application.* Journal of Pharmaceutical Sciences, 2003. **92**(2): p. 319-332.

73. Cleland, J.L., M.F. Powell, and S.J. Shire, *The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation.* Critical reviews in therapeutic drug carrier systems, 1993. **10**(4): p. 307-77.

74. Wang, W., S. Nema, and D. Teagarden, *Protein aggregation—Pathways and influencing factors.* International Journal of Pharmaceutics, 2010. **390**(2): p. 89-99.

75. Tsai, C.-J. and R. Nussinov, *Hydrophobic folding units at protein-protein interfaces: Implications to protein folding and to protein-protein association.* Protein Science, 1997. **6**(7): p. 1426-1437.

76. Azuaga, A.I., et al., *Unfolding and aggregation during the thermal denaturation of streptokinase.* European Journal of Biochemistry, 2002. **269**(16): p. 4121-4133.

77. Wedemeyer, W.J., et al., *Disulfide bonds and protein folding.* Biochemistry, 2000. **39**(15): p. 4207-4216.

78. Malencik, D.A. and S.R. Anderson, *Dityrosine as a product of oxidative stress and fluorescent probe.* Amino Acids, 2003. **25**(3-4): p. 233-247.

79. Trnkova, L., J. Drsata, and I. Bousova, *Oxidation as an important factor of protein damage: Implications for Maillard reaction.* Journal of Biosciences, 2015. **40**(2): p. 419-439.

80. Jiang, W.L. and S.P. Schwendeman, *Formaldehyde-mediated aggregation of protein antigens: Comparison of untreated and formalinized model antigens.* Biotechnology and Bioengineering, 2000. **70**(5): p. 507-517.

81. Murphy, R.M. and B.S. Kendrick, *Protein Misfolding and Aggregation.* Biotechnology Progress, 2007. **23**(3): p. 548-552.

82. Izutsu, K. and S. Kojima, *Freeze-concentration separates proteins and polymer excipients into different amorphous phases.* Pharmaceutical Research, 2000. **17**(10): p. 1316-1322.

83. Williams-Smith, D.L., et al., *Changes in apparent pH on freezing aqueous buffer solutions and their relevance to biochemical electron-paramagnetic-resonance spectroscopy.* Biochem J, 1977. **167**(3): p. 593-600.

84. Pikal-Cleland, K.A., et al., *Protein denaturation during freezing and thawing in phosphate buffer systems: Monomeric and tetrameric beta-galactosidase.* Archives of Biochemistry and Biophysics, 2000. **384**(2): p. 398-406.

85. Gómez, G., M. Pikal, and N. Rodríguez-Hornedo, *Effect of Initial Buffer Composition on pH Changes During Far-From-Equilibrium Freezing of Sodium Phosphate Buffer Solutions.* Pharmaceutical Research, 2001. **18**(1): p. 90-97.

86. Randolph, T.W., *Phase separation of excipients during lyophilization: Effects on protein stability.* Journal of Pharmaceutical Sciences, 1997. **86**(11): p. 1198-1203.

87. Sun, W.Q., et al., *Stability of dry liposomes in sugar glasses.* Biophysical Journal, 1996. **70**(4): p. 1769-1776.

88. Bieganski, R.M., et al., *Stabilization of active recombinant retroviruses in an amorphous dry state with trehalose.* Biotechnology Progress, 1998. **14**(4): p. 615-620.

89. Buitink, J. and O. Leprince, *Glass formation in plant anhydrobiotes: survival in the dry state.* Cryobiology, 2004. **48**(3): p. 215-228.

90. Allison, S.D., M.D.C. Molina, and T.J. Anchordoquy, *Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis.* Biochimica Et Biophysica Acta-Biomembranes, 2000. **1468**(1-2): p. 127-138.

91. Molina, M.D., et al., *The stability of lyophilized lipid/DNA complexes during prolonged storage.* Journal of Pharmaceutical Sciences, 2004. **93**(9): p. 2259-2273.

92. Carpenter, J.F. and J.H. Crowe, *An infrared spectroscopic study of the interactions of carbohydrates with dried proteins.* Biochemistry, 1989. **28**(9): p. 3916-3922.

93. Roos, Y. and M. Karel, *Plasticizing Effect of Water on Thermal Behavior and Crystallization of Amorphous Food Models.* Journal of Food Science, 1991. **56**(1): p. 38-43.

94. Chang, L., et al., *Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix?* Journal of Pharmaceutical Sciences, 2005. **94**(7): p. 1427-1444.

95. Crowe, J.H., J.F. Carpenter, and L.M. Crowe, *The role of vitrification in anhydrobiosis.* Annual Review of Physiology, 1998. **60**(1): p. 73-103.

96. Hatley, R.H.M. and J.A. Blair, *Stabilisation and delivery of labile materials by amorphous carbohydrates and their derivatives.* Journal of Molecular Catalysis B: Enzymatic, 1999. **7**(1–4): p. 11-19.

97. Gordon, M. and J.S. Taylor, *Ideal copolymers and the second-order transitions of synthetic rubbers. i. non-crystalline copolymers.* Journal of Applied Chemistry, 1952. **2**(9): p. 493-500.

98. Amann-Winkel, K., et al., *Water's second glass transition.* Proceedings of the National Academy of Sciences, 2013. **110**(44): p. 17720-17725.

99. Chen, T., A. Fowler, and M. Toner, *Literature Review: Supplemented Phase Diagram of the Trehalose–Water Binary Mixture.* Cryobiology, 2000. **40**(3): p. 277-282.

100. Hancock, B., S. Shamblin, and G. Zografi, *Molecular Mobility of Amorphous Pharmaceutical Solids Below Their Glass Transition Temperatures.* Pharmaceutical Research, 1995. **12**(6): p. 799-806.

101. Satvik Iyengar, A.R., et al., *Improving storage stability of recombinant organophosphorus hydrolase.* Protein Expression and Purification, 2015. **111**: p. 28-35.

102. Lee, Y.-C., et al., *The protective effect of lactose on lyophilization of CNK-20402.* AAPS PharmSciTech, 2005. **6**(1): p. E42-E48.

103. Allison, S.D., et al., *Hydrogen Bonding between Sugar and Protein Is Responsible for Inhibition of Dehydration-Induced Protein Unfolding.* Archives of Biochemistry and Biophysics, 1999. **365**(2): p. 289-298.

104. Roos, Y.H. and N. Potes, *Quantification of Protein Hydration, Glass Transitions, and Structural Relaxations of Aqueous Protein and Carbohydrate–Protein Systems.* The Journal of Physical Chemistry B, 2015. **119**(23): p. 7077-7086.

105. Anchordoquy, T.J., et al., *Maintenance of Quaternary Structure in the Frozen State Stabilizes Lactate Dehydrogenase during Freeze–Drying.* Archives of Biochemistry and Biophysics, 2001. **390**(1): p. 35-41.

106. Souillac, P.O., et al., *Investigation of protein/carbohydrate interactions in the dried state. 1. Calorimetric studies.* Journal of Pharmaceutical Sciences, 2002. **91**(1): p. 206-216.

107. Allison, S.D., et al., *Optimization of storage stability of lyophilized actin using combinations of disaccharides and dextran.* Journal of Pharmaceutical Sciences, 2000. **89**(2): p. 199-214.

108. Amorij, J.P., et al., *Pulmonary delivery of an inulin-stabilized influenza subunit vaccine prepared by spray-freeze drying induces systemic, mucosal humoral as well as cell-mediated immune responses in BALB/c mice.* Vaccine, 2007. **25**(52): p. 8707-8717.

109. Hincha, D.K., et al., *Plant fructans stabilize phosphatidylcholine liposomes during freeze-drying.* European Journal of Biochemistry, 2000. **267**(2): p. 535-540.

110. Li, S., et al., *Effects of reducing sugars on the chemical stability of human relaxin in the lyophilized state.* Journal of Pharmaceutical Sciences, 1996. **85**(8): p. 873-877.

111. Carpenter, J.F., et al., *Rational design of stable lyophilized protein formulations: some practical advice.* Pharmaceutical Research, 1997. **14**(8): p. 969-975.

112. Zhang, Y.J. and P.S. Cremer, *Interactions between macromolecules and ions: the Hofmeister series.* Current Opinion in Chemical Biology, 2006. **10**(6): p. 658-663.

113. Der, A., et al., *Interfacial water structure controls protein conformation.* Journal of Physical Chemistry B, 2007. **111**(19): p. 5344-5350.

114. Arakawa, T. and S.N. Timasheff, *Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding.* Biochemistry, 1984. **23**(25): p. 5912-5923.

115. Zhang, Y.J. and P.S. Cremer, *Chemistry of Hofmeister Anions and Osmolytes*, in *Annual Review of Physical Chemistry, Vol 61*, S.R. Leone, et al., Editors. 2010, Annual Reviews: Palo Alto. p. 63-83.

116. Cacace, M.G., E.M. Landau, and J.J. Ramsden, *The Hofmeister series: salt and solvent effects on interfacial phenomena.* Quarterly Reviews of Biophysics, 1997. **30**(03): p. 241-277.

117. Shikama, K. and T. Yamazaki, *Denaturation of Catalase by Freezing and Thawing.* Nature, 1961. **190**(4770): p. 83-84.

118. Andersson, M.M. and R. Hatti-Kaul, *Protein stabilising effect of polyethyleneimine.* Journal of Biotechnology, 1999. **72**(1–2): p. 21-31.

119. Ohtake, S., Y. Kita, and T. Arakawa, *Interactions of formulation excipients with proteins in solution and in the dried state.* Adv Drug Deliv Rev, 2011. **63**(13): p. 1053-73.

120. Crowe, J.H., L.M. Crowe, and J.F. Carpenter, *Preserving dry biomaterials: the water replacement hypothesis.* BioPharm, 1993. **6**: p. 40-43.

121. Imamura, K., et al., *Water sorption, glass transition, and protein-stabilizing behavior of an amorphous sucrose matrix combined with various materials.* Journal of Pharmaceutical Sciences, 2010. **99**(11): p. 4669-4677.

122. Choi, H.-J., et al., *Effect of Osmotic Pressure on the Stability of Whole Inactivated Influenza Vaccine for Coating on Microneedles.* PLoS ONE, 2015. **10**(7): p. e0134431.

123. Rexroad, J., et al., *Lyophilization and the Thermostability of Vaccines.* Cell Preservation Technology, 2002. **1**(2): p. 91-104.

124. Lovett, S.T., *Encoded errors: mutations and rearrangements mediated by misalignment at repetitive DNA sequences.* Mol Microbiol, 2004. **52**(5): p. 1243-53.

125. Lechardeur, D., A.S. Verkman, and G.L. Lukacs, *Intracellular routing of plasmid DNA during non-viral gene transfer.* Adv Drug Deliv Rev, 2005. **57**(5): p. 755-67.

126. Goldblatt, D., *Conjugate vaccines.* Clinical and Experimental Immunology, 2000. **119**(1): p. 1-3.

127. Pichichero, M.E., *Protein carriers of conjugate vaccines Characteristics, development, and clinical trials.* Human Vaccines & Immunotherapeutics, 2013. **9**(12): p. 2505-2523.

128. Corbel, M.J., *Reasons for instability of bacterial vaccines*, in *New Approaches to Stabilisation of Vaccines Potency*, F. Brown, Editor. 1996, Karger: Basel. p. 113-124.

129. PATH Org. *Stabilizing influenza vaccines*. 2013; Available from: http://www.path.org/publications/files/TS_vtg_siv_fs.pdf.

130. Patois, E., et al., *Stability of seasonal influenza vaccines investigated by spectroscopy and microscopy methods.* Vaccine, 2011. **29**(43): p. 7404-7413.

131. Amorij, J.P., et al., *Rational design of an influenza subunit vaccine powder with sugar glass technology: Preventing conformational changes of haemagglutinin during freezing and freeze-drying.* Vaccine, 2007. **25**(35): p. 6447-6457.

132. Chen, D., et al., *Epidermal powder immunization of mice and monkeys with an influenza vaccine.* Vaccine, 2003. **21**(21–22): p. 2830-2836.

133. Maa, Y.-F., et al., *Influenza vaccine powder formulation development: spray-freeze-drying and stability evaluation.* Journal of Pharmaceutical Sciences, 2004. **93**(7): p. 1912-1923.

134. Chen, D.X., et al., *Epidermal immunization by a needle-free powder delivery technology: Immunogenicity of influenza vaccine and protection in mice.* Nature Medicine, 2000. **6**(10): p. 1187-1190.

135. Huang, J., et al., *A novel dry powder influenza vaccine and intranasal delivery technology: induction of systemic and mucosal immune responses in rats.* Vaccine, 2004. **23**(6): p. 794-801.

136. de Jonge, J., et al., *Inulin sugar glasses preserve the structural integrity and biological activity of influenza virosomes during freeze-drying and storage.* European Journal of Pharmaceutical Sciences, 2007. **32**(1): p. 33-44.

137. Moldoveanu, Z., et al., *Human immune responses to influenza virus vaccines administered by systemic or mucosal routes.* Vaccine, 1995. **13**(11): p. 1006-1012.

138. Quan, F.S., et al., *Stabilization of Influenza Vaccine Enhances Protection by Microneedle Delivery in the Mouse Skin.* Plos One, 2009. **4**(9).

139. Kim, Y.C., et al., *Stability kinetics of influenza vaccine coated onto microneedles during drying and storage.* Pharmaceutical Research, 2011. **28**(1): p. 135-144.

140. Kommareddy, S., et al., *Influenza subunit vaccine coated microneedle patches elicit comparable immune responses to intramuscular injection in guinea pigs.* Vaccine, 2013. **31**(34): p. 3435-3441.

141. Chen, X.F., et al., *Rapid kinetics to peak serum antibodies is achieved following influenza vaccination by dry-coated densely packed microprojections to skin.* Journal of Controlled Release, 2012. **158**(1): p. 78-84.

142. Hirobe, S., et al., *Clinical study and stability assessment of a novel transcutaneous influenza vaccination using a dissolving microneedle patch.* Biomaterials, 2015. **57**(0): p. 50-58.

143. Gerdts, V., et al., *Use of animal models in the development of human vaccines.* Future Microbiology, 2007. **2**(6): p. 667-75.

144. Luke, C.J. and K. Subbarao, *The Role of Animal Models In Influenza Vaccine Research*, in *Influenza Vaccines for the Future, Second Edition*, R. Rappuoli and G. DelGiudice, Editors. 2011, Birkhauser Verlag Ag: Basel. p. 223-272.

145. WHO Collaborating Centers for Reference and Research on Influenza, *Concepts and procedures for laboratory-based influenza surveillance*. 1982.

146. Flint, S., et al., *Virological Methods*, in *Principles of Virology*. 2009, ASM Press.

147. Chen, M.H., et al., *An indirect immunocolorimetric assay to detect rubella virus infected cells.* Journal of Virological Methods, 2007. **146**(1-2): p. 414-418.

148. Gan, S.D. and K.R. Patel, *Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay.* J Invest Dermatol, 2013. **133**(9): p. e12.

149. Xu, R.L., *Light scattering: A review of particle characterization applications.* Particuology, 2015. **18**: p. 11-21.

150. World Health Organization. *Biologicals - Influenza*. 2014 11/4/15]; Available from: http://www.who.int/biologicals/vaccines/influenza/en/.

151. Kissmann, J., et al., *Stabilization of measles virus for vaccine formulation.* Human Vaccines, 2008. **4**(5): p. 350-359.

152. Ruigrok, R.W.H., et al., *Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes.* Virology, 1986. **155**(2): p. 484-497.

153. Luykx, D.M.A.M., et al., *Physicochemical studies on the stability of influenza haemagglutinin in vaccine bulk material.* European Journal of Pharmaceutical Sciences, 2004. **23**(1): p. 65-75.

154. Katz, A., et al. *Fluorescence of Influenza Hemagglutinin Surface Protein*. in *Frontiers in Optics 2011/Laser Science XXVII*. 2011. San Jose, California: Optical Society of America.

155. Receveur-Bréchot, V., et al., *Assessing protein disorder and induced folding.* Proteins: Structure, Function, and Bioinformatics, 2006. **62**(1): p. 24-45.

156. Ghisaidoobe, A.B.T. and S.J. Chung, *Intrinsic Tryptophan Fluorescence in the Detection and Analysis of Proteins: A Focus on Förster Resonance Energy Transfer Techniques.* International Journal of Molecular Sciences, 2014. **15**(12): p. 22518-22538.

157. Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*. 3rd ed. 2006: Springer.

158. Kelly, S.M. and N.C. Price, *The use of circular dichroism in the investigation of protein structure and function.* Curr Protein Pept Sci, 2000. **1**(4): p. 349-84.

159. Sreerama, N., S.Y. Venyaminov, and R.W. Woody, *Estimation of Protein Secondary Structure from Circular Dichroism Spectra: Inclusion of Denatured Proteins with Native Proteins in the Analysis.* Analytical Biochemistry, 2000. **287**(2): p. 243-251.

160. World Health Organization. *Influenza (Seasonal) Fact Sheet*. 2014 10/24/15]; Available from: http://www.who.int/mediacentre/factsheets/fs211/en/index.html.

161. Grohskopf, L. and et. al., *Prevention and control of seasonal influenza with vaccines: Recommendations of the Advisory Committee on Immunization Practices (ACIP) — United States, 2014–15 Influenza Season.* Morbidity and Mortality Weekly Report, 2014. **63**: p. 691-697.

162. Santibanez TA, et al. *Flu Vaccination Coverage, United States, 2012-13 Influenza Season*. 2013 7/1/14]; Available from: http://www.cdc.gov/flu/fluvaxview/coverage-1213estimates.htm.

163. Taddio, A., et al., *Survey of the prevalence of immunization non-compliance due to needle fears in children and adults.* Vaccine, 2012. **30**(32): p. 4807-12.

164. Kommareddy, S., et al., *Dissolvable microneedle patches for the delivery of cell-culture-derived influenza vaccine antigens.* Journal of Pharmaceutical Sciences, 2012. **101**(3): p. 1021-1027.

165. Kang, S.M., J.M. Song, and Y.C. Kim, *Microneedle and mucosal delivery of influenza vaccines.* Expert Review of Vaccines, 2012. **11**(5): p. 547-60.

166. Matsuo, K., et al., *Transcutaneous immunization using a dissolving microneedle array protects against tetanus, diphtheria, malaria, and influenza.* Journal of Controlled Release, 2012. **160**(3): p. 495-501.

167. Koutsonanos, D., R. Compans, and I. Skountzou, *Targeting the skin for microneedle delivery of influenza vaccine*, in *Crossroads Between Innate and Adaptive Immunity IV*, P.D. Katsikis, S.P. Schoenberger, and B. Pulendran, Editors. 2013, Springer New York. p. 121-132.

168. Norman, J.J., et al., *Microneedle patches: Usability and acceptability for self-vaccination against influenza.* Vaccine, 2014. **32**(16): p. 1856-1862.

169. Pikal, M.J., *Mechanisms of protein stabilization during freeze-drying storage: The relative protection by stabilizing additives*, in *Freeze-drying/lyophilization of pharmaceutical and biological products*, L. Rey and J.C. May, Editors. 2010, CRC Press.

170. Kim, Y.C., et al., *Formulation and coating of microneedles with inactivated influenza virus to improve vaccine stability and immunogenicity.* Journal of Controlled Release, 2010. **142**(2): p. 187-195.

171. Chen, X., et al., *Improving the reach of vaccines to low-resource regions, with a needle-free vaccine delivery device and long-term thermostabilization.* Journal of Controlled Release, 2011. **152**(3): p. 349-355.

172. Choi, H.J., et al., *Stability of whole inactivated influenza virus vaccine during coating onto metal microneedles.* Journal of Controlled Release, 2013. **166**(2): p. 159-71.

173. Caligur, V., *Detergent properties and applications.* BioFiles, 2008. **3**(3): p. 14.

174. Nazari, M., M. Kurdi, and H. Heerklotz, *Classifying surfactants with respect to their effect on lipid membrane order.* Biophysical Journal, 2012. **102**(3): p. 498-506.

175. Schlehuber, L.D., et al., *Towards ambient temperature-stable vaccines: The identification of thermally stabilizing liquid formulations for measles virus using an innovative high-throughput infectivity assay.* Vaccine, 2011. **29**(31): p. 5031-5039.

176. Lee, J.W., J.H. Park, and M.R. Prausnitz, *Dissolving microneedles for transdermal drug delivery.* Biomaterials, 2008. **29**(13): p. 2113-2124.

177. Williams, R. and C. Lyman, *A neutral buffered standard for hydrogen ion work and accurate titrations which can be prepared in one minute.* Journal of the American Chemical Society, 1932. **54**(5): p. 1911-1912.

178. Wolfson LJ, et al., *Estimating the costs of achieving the WHO-UNICEF Global Immunization Vision and Strategy, 2006-2015.* Bulletin of the World Health Organization, 2008. **86**(1): p. 27-39.

179. Brandau, D.T., et al., *Thermal stability of vaccines.* Journal of Pharmaceutical Sciences, 2003. **92**(2): p. 218-231.

180. CDC. *Flu Vaccination Coverage, United States, 2014-15 Influenza Season*. 2015 [cited 2015 December]; Available from: http://www.cdc.gov/flu/fluvaxview/coverage-1415estimates.htm.

181. Das, P., *Revolutionary vaccine technology breaks the cold chain.* The Lancet Infectious Diseases, 2004. **4**(12): p. 719.

182. Cicerone, M.T. and C.L. Soles, *Fast dynamics and stabilization of proteins: binary glasses of trehalose and glycerol.* Biophysical Journal, 2004. **86**(6): p. 3836-3845.

183. Chang, L. and M.J. Pikal, *Mechanisms of protein stabilization in the solid state.* Journal of Pharmaceutical Sciences, 2009. **98**(9): p. 2886-2908.

184. Lee, V.H.L., *Changing needs in drug delivery in the era of peptide and protein drugs*, in *Peptide and Protein Drug Delivery*, V.H.L. Lee, Editor. 1991, Marcel Dekker, Inc.: New York. p. 1-56.

185. Pearton, M., et al., *Immune stimulation following microneedle delivery of influenza virus-like particle (VLP) vaccines to human skin.* Drug Discovery Today, 2010. **15**(23-24): p. A87.

186. Donnelly, R.F., T.R.R. Singh, and A.D. Woolfson, *Microneedle-based drug delivery systems: Microfabrication, drug delivery, and safety.* Drug Delivery, 2010. **17**(4): p. 187-207.

187. Zhang, J., et al., *Stabilization of vaccines and antibiotics in silk and eliminating the cold chain.* Proceedings of the National Academy of Sciences, 2012.

188. Kim, Y.C., et al., *Enhanced memory responses to seasonal H1N1 influenza vaccination of the skin with the use of vaccine-coated microneedles.* Journal of Infectious Diseases, 2010. **201**(2): p. 190-198.

189. Wang, W., *Lyophilization and development of solid protein pharmaceuticals.* International Journal of Pharmaceutics, 2000. **203**(1-2): p. 1-60.

190. Cicerone, M.T., M.J. Pikal, and K.K. Qian, *Stabilization of proteins in solid form.* Advanced Drug Delivery Reviews, 2015. **93**: p. 14-24.

191. Crowe, L.M., D.S. Reid, and J.H. Crowe, *Is trehalose special for preserving dry biomaterials?* Biophysical Journal, 1996. **71**(4): p. 2087-2093.

192. Grasmeijer, N., et al., *Unraveling protein stabilization mechanisms: Vitrification and water replacement in a glass transition temperature controlled system.* Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 2013. **1834**(4): p. 763-769.

193. Monkos, K., *Studies of protein solution conformations using viscometric measurements*, in *Conformational Stability, Size, Shape and Surface of Protein Molecules*, V.N. Uversky and E.A. Permyakov, Editors. 2007, Nova Science Publishers. p. 363.

194. Geeraedts, F., et al., *Preservation of the immunogenicity of dry-powder influenza H5N1 whole inactivated virus vaccine at elevated storage temperatures.* The AAPS Journal, 2010. **12(**2): p. 215-222.

195. Mistilis, M.J., A.S. Bommarius, and M.R. Prausnitz, *Development of a thermostable microneedle patch for influenza vaccination.* Journal of Pharmaceutical Sciences, 2015. **104**(2): p. 740-749.

196. Chu, L.Y., S.O. Choi, and M.R. Prausnitz, *Fabrication of dissolving polymer microneedles for controlled drug encapsulation and delivery: bubble and pedestal microneedle designs.* Journal of Pharmaceutical Sciences, 2010. **99**(10): p. 4228-4238.

197. Koutsonanos, D.G., et al., *Delivery of subunit influenza vaccine to skin with microneedles improves immunogenicity and long-lived protection.* Scientific Reports, 2012. **2**.

198. Crowe, J.H., B.J. Spargo, and L.M. Crowe, *Preservation of dry liposomes does not require retention of residual water.* Proc Natl Acad Sci U S A, 1987. **84**(6): p. 1537-40.

199. Costantino, H.R., et al., *Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone.* Journal of Pharmaceutical Sciences, 1998. **87**(11): p. 1412-1420.

200. Mattern, M., et al., *Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems.* Pharmaceutical Development And Technology, 1999. **4**(2): p. 199-208.

201. Hai, T.T. and D.J. Nelson, *Stabilization of therapeutic hemoglobin compositions*, USPTO, Editor. 1997.

202. Roe, K.D. and T.P. Labuza, *Glass transition and crystallization of amorphous trehalose-sucrose mixtures.* International Journal of Food Properties, 2005. **8**(3): p. 559-574.

203. Wolfe, J., G. Bryant, and K.L. Koster, *What is 'unfreezable water', how unfreezable is it, and how much is there?* Cryo Letters, 2002. **23**(3): p. 157-66.

204. Ameri, M., X.M. Wang, and Y.F. Maa, *Effect of irradiation on parathyroid hormone PTH(1-34) coated on a novel transdermal microprojection delivery system to produce a sterile product-adhesive compatibility.* Journal of Pharmaceutical Sciences, 2010. **99**(4): p. 2123-2134.

205. Williams, M.S., *Single-radial-immunodiffusion as an in vitro potency assay for human inactivated viral vaccines.* Veterinary Microbiology, 1993. **37**(3-4): p. 253-262.

206. Skountzou, I. and R.W. Compans, *Skin immunization with influenza vaccines.* Curr Top Microbiol Immunol, 2015. **386**: p. 343-69.

207. World Health Organization. *Influenza Fact Sheet*. 2014 10/24/15]; Available from: http://www.who.int/mediacentre/factsheets/2003/fs211/en/.

208. Centers for Disease Control and Prevention. *Seasonal Influenza Vaccine Supply for the U.S. 2015-2016 Influenza Season*. 2015 [cited 2015 10/24/15]; Available from: http://www.cdc.gov/flu/about/qa/vaxsupply.htm.

209. *Storage and Handling*, in *Epidemiology and Prevention of Vaccine-Preventable Diseases*, J. Hamborsky, A. Kroger, and C. Wolfe, Editors. 2015, Centers for Disease Control and Prevention. p. 63-77.

210. National Institutes of Health. *Inactivated Influenza Vaccine Delivered by Microneedle Patch or by Hypodermic Needle*. [cited 2015; Available from: https://clinicaltrials.gov/ct2/show/NCT02438423.

211. Constantino, H.R., *Excipients for Use in Lyophilized Pharmaceutical Peptide, Protein, and other Bioproducts*, in *Lyophilization of biopharmaceuticals*, H.R. Constantino and M.J. Pikal, Editors. 2004, American Association of Pharmaceutical Scientists: Arlington, VA. p. 140.

212. Kasper, J.C. and W. Friess, *The freezing step in lyophilization: Physico-chemical fundamentals, freezing methods and consequences on process performance and quality attributes of biopharmaceuticals.* European Journal of Pharmaceutics and Biopharmaceutics, 2011. **78**(2): p. 248-263.

213. Steinhauer, D.A., *Role of Hemagglutinin Cleavage for the Pathogenicity of Influenza Virus.* Virology, 1999. **258**(1): p. 1-20.

214. Korte, T., et al., *Conformational change of influenza virus hemagglutinin is sensitive to ionic concentration.* European Biophysics Journal, 2007. **36**(4-5): p. 327-335.

215. Huang, Q., et al., *Protonation and stability of the globular domain of influenza virus hemagglutinin.* Biophysical Journal, 2002. **82**(2): p. 1050-1058.

216. Soema, P.C., et al., *Solid Bioneedle-Delivered Influenza Vaccines Are Highly Thermostable and Induce Both Humoral and Cellular Immune Responses.* Plos One, 2014. **9**(3).

217. Ruigrok, R.W., et al., *Electron microscopy of the low pH structure of influenza virus haemagglutinin.* The EMBO Journal, 1986. **5**(1): p. 41-49.

218. Wilson, I.A., J.J. Skehel, and D.C. Wiley, *Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 [angst] resolution.* Nature, 1981. **289**(5796): p. 366-373.

219. Remeta, D.P., et al., *Acid-Induced Changes in Thermal Stability and Fusion Activity of Influenza Hemagglutinin.* Biochemistry, 2002. **41**(6): p. 2044-2054.

220. Gunasegaran, K., *FIELDS VIROLOGY, 4TH EDITION.* Immunol Cell Biol, 2002. **80**(3): p. 314-315.

221. de Xammar Oro, J., *Role of Co-Solute in Biomolecular Stability: Glucose, Urea and the Water Structure.* Journal of Biological Physics, 2001. **27**(1): p. 73-79.

222. Uedaira, H. and H. Uedaira, *Role of hydration of polyhydroxy compounds in biological systems.* Cellular and Molecular Biology, 2001. **47**(5): p. 823-829.

223. Magazù, S., F. Migliardo, and A.J. Ramirez-Cuesta, *Kosmotrope character of maltose in water mixtures.* Journal of Molecular Structure, 2007. **830**(1–3): p. 167-170.

224. Curtis, R.A., et al., *Protein–protein interactions in concentrated electrolyte solutions.* Biotechnology and Bioengineering, 2002. **79**(4): p. 367-380.

225. Yu, I. and M. Nagaoka, *Slowdown of water diffusion around protein in aqueous solution with ectoine.* Chemical Physics Letters, 2004. **388**(4–6): p. 316-321.

226. Wharton, S.A., et al., *Conformational aspects of the acid-induced fusion mechanism of influenza virus hemagglutinin. Circular dichroism and fluorescence studies.* J Biol Chem, 1988. **263**(9): p. 4474-80.

227. Ranjbar, B. and P. Gill, *Circular Dichroism Techniques: Biomolecular and Nanostructural Analyses- A Review.* Chemical Biology & Drug Design, 2009. **74**(2): p. 101-120.

228. Zaffran, M., et al., *The imperative for stronger vaccine supply and logistics systems.* Vaccine, 2013. **31**: p. B73-B80.

229. Mukuta, T., et al., *Influence of Impurities on the Solution-Mediated Phase Transformation of an Active Pharmaceutical Ingredient.* Crystal Growth & Design, 2005. **5**(4): p. 1429-1436.

230. Burke, C.J., T.A. Hsu, and D.B. Volkin, *Formulation, stability, and delivery of live attenuated vaccines for human use.* Critical Reviews in Therapeutic Drug Carrier Systems, 1999. **16**(1): p. 1-83.

231. Zeng, Y., et al., *Identifying stabilizers of plasmid DNA for pharmaceutical use.* Journal of Pharmaceutical Sciences, 2011. **100**(3): p. 904-914.

232. Jacoby, E., et al., *Opportunities and challenges in delivering influenza vaccine by microneedle patch.* Vaccine, 2015. **33**(37): p. 4699-4704.

233. Ling, M.-H. and M.-C. Chen, *Dissolving polymer microneedle patches for rapid and efficient transdermal delivery of insulin to diabetic rats.* Acta Biomaterialia, 2013. **9**(11): p. 8952-8961.